Parasite Distribution and Specificity in Anuran Host Complexes.

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Abstract.

This study was based on the parasite fauna of three anuran systems. In two of the systems; the spadefoot toads, *Scaphiopus bombifrons* and *S.multiplicatus* and the fire and yellow-bellied toads, *Bombina bombina* and *B.variegata*, inter-species hybridisation occurs. In the third system an autopolyploid species complex involves the Gray treefrogs, *Hyla chrysoscelis* and *H.versicolor*. The status of the *Scaphiopus* hybrid zone in Arizona was re-assessed by cellulose-acetate gel electrophoresis. Genotypic frequencies were comparable to those previously documented, indicating that a region of 'balanced introgression' may exist.

Specimens were collected in areas of host sympatry and the distribution of helminths within hosts of different genetic constitution investigated. From extensive surveys of all three systems, parasite frequency distributions were typically aggregated with no evidence of any change in host genetic predisposition to infection. In addition, the surveys have documented a number of new host and/or parasite locality records for each system. Of particular interest was the discovery of the digenean *Clinostomum complanatum* in a desert environment, an introduction related to human management of the ecosystem.

Particular reference was made to the specificity of the polystomatid monogeneans, *Polystoma nearcticum* infecting members of the *H.chrysoscelis-H.versicolor* complex and *Neodiplorchis scaphiopodis* infecting *S.bombifrons* and *S.multiplicatus*. For *Polystoma*, worms from *H.chrysoscelis* and *H.versicolor* could not be separated by morphology, which was supported by preliminary cross-infections. This suggests that *P.nearcticum* infects both members of this complex. For *Neodiplorchis*, morphological similarity was confirmed by cross-infections which revealed that *N.scaphiopodis* can reach sexual maturity and produce fully developed larvae in heterospecific hosts.

The neotenic development of *P.nearcticum* and the initial post-oncomiracidial stages of *N.scaphiopodis* were documented. Furthermore, certain aspects of transmission dynamics were examined by the experimental infection of *H.versicolor* tadpoles with *P.nearcticum*.

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Chapter 1.

1.1 General Introduction.

This project was based on the parasite fauna of three anuran systems. In two of the systems; the North American spadefoot toads, *Scaphiopus bombifrons* and *S.multiplicatus*, and the European fire and yellow-bellied toads, *Bombina bombina* and *B.variegata*, there is a breakdown in reproductive isolation between the host species, leading to introgression within zones of hybridisation. In both cases, hybrid toads are sympatric with pure host species, thus presenting parasites with an array of genotypes. The third system concerns the North American Gray treefrogs, *Hyla chrysoscelis* and *H.versicolor*, which form a polyploid species complex. The complex is thought to have multiple origins, speciation having occurred via autopolyploidy, confronting parasites with an instantaneous doubling of the host genome. The systems are described below with a brief introduction to the theoretical background.

Barton & Hewitt (1985) defined hybrid zones as 'narrow regions in which genetically distinct populations meet, mate and produce hybrids'. These zones are of great interest as they challenge many published definitions of species and speciation (Dobzhansky, 1940; Mayr, 1942). Mayr (1942) defined a biological species as a 'group of actually or potentially interbreeding natural populations which are reproductively isolated from other such groups'. Further qualifications and variations have subsequently been made (Bigelow, 1965; Grant, 1978),

however, Barton & Hewitt (1989) argued that the originators of the biological species concept had classed taxa, which are exposed to gene flow and yet remain distinct, as separate species.

There are numerous hybrid zones documented in the literature (see reviews by Barton & Hewitt, 1985, 1989; Moore, 1977). For example, hybridisation between anurans is common in the United States, including *Bufo* (Feder, 1973; Green, 1984; Jones, 1973; Volpe, 1952), *Rana* (Brown, 1973; Frost & Bagnara, 1977; Frost & Platz, 1983; Hillis, 1981; Platz, 1972; Sage & Selander, 1979) and *Scaphiopus* (Sattler, 1985; Simovich, 1986; Wasserman, 1957).

The closely related *S.bombifrons* and *S.multiplicatus* are members of the amphibian family Pelobatidae. They are found exclusively in the Nearctic, their ranges overlapping in the southern Great Plains of Arizona, Colorado, New Mexico, Oklahoma, Texas and northern Mexico (Stebbins, 1985). In regions of sympatry, a number of zones of hybridisation have been recorded (Brown, 1976; Forester, 1973; Hughes, 1965; Sattler, 1978), although the particular area to be examined in this study was a spatially and temporally dynamic zone in Arizona (Simovich, 1986). *S.bombifrons* is generally considered to be a plains species (Stebbins, 1985), inhabiting lower elevation grasslands and river valleys (Tanner, 1989; Wasserman, 1964). *S.multiplicatus* favours the desert scrub at higher elevations, being less abundant in grassland areas (Ruibal, Tevis & Roig, 1969; Tanner, 1989).

The European fire and yellow-bellied toads, *B.bombina* and *B.variegata*, form a narrow hybrid zone from Poland to the Black Sea, where elevations lie between the preferred habitats of pure-bred populations. Hybrid zones have been mapped in Austria, Bulgaria, Czechoslovakia, Hungary, Poland and Yugoslavia (Gollmann, 1984, 1986, 1987; Gollmann, Roth & Hodl, 1988; Szymura 1976a, 1976b, 1988; Szymura & Barton, 1986, 1991). Although closely related (Szymura, 1988), there are marked differences in the ecology of the toads. *B.bombina* is a lowland species found in northern and central Europe and *B.variegata* is found in the upper elevations of western, central and southern Europe (Arntzen, 1978). Typically, *B.bombina* lives in the vicinity of large water courses and breeding is prolonged. Inhabiting more montane regions, *B.variegata* is relatively more terrestrial, tending to utilise small or temporary pools which are prone to drying (Barandun, 1991; Medej, 1973; Rafinska, 1991).

The third host group are the Gray treefrogs, which are native to the woodlands of the mid-west and eastern United States. They form a cryptic species pair, distinguished by mating call and chromosome number, *H.chrysoscelis* (2n = 24) is the diploid progenitor of the tetraploid *H.versicolor* (2n = 48) (Wasserman, 1970). Recent studies suggest that there are three independent origins of the tetraploid via autopolyploidy: the spontaneous occurrence of chromosome doubling (Ptacek, Gerhardt & Sage, 1993). Reproductive isolation is maintained by strong female selection, with hybrids forming less than 0.05% of sympatric populations (Gerhardt *et al.*, 1994). Ralin (1968) proposed that, in sympatry, there may also be ecological segregation in food preference. However both species of Gray

treefrog are typically found in small wooded plots close to water courses. They are nocturnal, feeding on invertebrate prey either on the ground or in the arboreal vegetation. Strong site-fidelity has been demonstrated throughout the year, with little movement between locations (Ritke & Babb, 1991; Ritke, Babb & Ritke, 1990, 1991).

Parasites are typically aggregated within host populations, generally fitting an overdispersed distribution (Crofton, 1971). The influence of host genetics in determining the levels of parasitic infection was examined by detailed parasitological surveys of all three host systems. However, particular reference was made to the polystomatid monogeneans, *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 infecting *S.bombifrons* and *S.multiplicatus*, and *Polystoma nearcticum* (Paul, 1935) Price, 1939 infecting members of the *H.chrysoscelis-versicolor* complex.

Monogeneans are hermaphroditic flatworms possessing a direct life-cycle, transmission being effected by a ciliated, aquatic larva. They are typically external parasites of vertebrates, most commonly recovered from piscine hosts. Internal habitats include the urinary bladders of chelonian reptiles, anuran and urodele amphibians (Prudhoe & Bray, 1982). Both *N.scaphiopodis* and *P.nearcticum* are members of the family Polystomatidae Carus, 1863. Representatives of the Polystomatidae exhibit a number of features which are unprecedented in parasitology (Tinsley, 1993) and have been recovered from all continents, except Antarctica.

Tinsley & Earle (1983) documented the unique life-cycle of *N.scaphiopodis* and the closely related *P.americanus*. The almost exclusively terrestrial ecology of the host restricts opportunities for transmission to the brief breeding season, the only aquatic phase of the toad's life-cycle. Breeding congregations are triggered by torrential rainfall forming numerous ephemeral ponds in the desert. Characteristically, the toads breed on the first night after the onset of rain, leaving the water at dawn. This reduces the aquatic phase to a maximum of 7 hours on any one occasion. In any given season, males may enter breeding congregations on 1-3 occasions but females only mate once. Therefore *N.scaphiopodis* has a maximum window for infection of 24h/toad/year (Tinsley, 1989). *N.scaphiopodis* is adapted to meet these restrictions by accumulating offspring *in utero* during host hibernation and releasing immediately infective larvae into the water. The trigger for release is thought to be related to host sexual excitement (Tinsley, 1990). The high population densities of both hosts and parasite larvae contribute to very effective transmission. Oncomiracidia enter the respiratory tract via the nostrils, and migrate to the lungs. The utilisation of the lungs remains unprecedented in monogenean biology (Tinsley & Earle, 1983). The worm's route to the bladder involves migrating through the alimentary tract, with host activity providing the stimulus to migrate (Tinsley & Jackson, 1986). Those worms which reach maturity are ready to release offspring of their own when their host next enters the water, a year later.

The reproductive strategy of *P.nearcticum* is also closely linked to the complex behaviour of the host. The deposition of eggs from adult worms is restricted to

periods when the host enters water, for example on breeding nights during the two month breeding season (Hausfater *et al.*, 1990). This release is also believed to be related to host sexual excitement (Tinsley, 1991). Invasion is restricted to the larval stage of the host's life-cycle, with recently established worms exhibiting dimorphic developmental paths. The 'neotenics' are sexually mature forms which rapidly develop on the tadpole gills to produce eggs at 2-4 weeks post-infection, dying at host metamorphosis (Gallien, 1935; Combes, 1968). Neotenics augment the punctuated deposition of adult-derived eggs with a steady 'trickle' infection, which may boost invasion levels immediately prior to metamorphosis (Savage, 1950). Also, typically inhabiting the same host are the 'bladder-destined' forms which exhibit a much slower developmental rate, moving from the gills to the urinary bladder at host metamorphosis (Combes, 1967). Although not fully established, it has been suggested that the biochemical status of the host at the time of oncomiracidial invasion determines the developmental path taken (Murith, 1982).

The recovery of *N.scaphiopodis* from two host species (Rodgers, 1941; Lamothe-Argumedo, 1973) and reports of *P.nearcticum* from three host species (*H.cinerea*, *H.squirella* & *H.versicolor*) (Paul, 1938; Price, 1939) is of great interest as strict host specificity within the anuran Polystomatidae has been proposed by a number of authors (Bourgat, 1977; Bourgat & Salami-Cadoux, 1976; Combes & Channing, 1978-1979; Euzet, Combes & Knoepffler, 1966; Kok & van Wyk, 1986; Murith, 1981). This assumption was mainly based on studies of African *Polystoma*, species delineation centring on morphological variation,

sparse experimental evidence (Combes, 1966, 1968) and unpublished cross-infections of tadpoles referred to in papers by Bourgat & Salami-Cadoux (1976) and Kok & DuPreez (1993). Moreover, a broader host specificity has been recorded by a number of authors for branchial worms (Kok & DuPreez, 1987; Maeder, 1973) and adults (Murith, 1981; Vaucher, 1990). The sympatric occurrence of two species in a single host species (Bourgat & Murith, 1980) and the syntopic occurrence two genera within the same host (DuPreez & Kok, 1992) has also been documented. Prudhoe & Bray (1982) commented that morphological differentiation must be arbitrary if identification of the parasite could not be made without knowledge of its host. These taxonomic problems return to the basic tenet regarding both host and parasite, that is species definition. No comparative studies of polystomatids occurring in the nearctic have been made, with only species and locality records present in the literature.

A diverse parasite fauna has been recorded from *B.bombina*, *B.variegata* and their hybrids. This study centres on specimens collected in the southern portion of the host hybrid zone, in the former Yugoslavia. The sample provides an opportunity to examine the infection levels of all helminths between pure and hybrid hosts in this region. The influence of host genetics on susceptibility to parasitic infection is of particular interest as field studies of wild populations of mice in Europe have documented overwhelming helminth burdens in individuals of hybrid genotype compared to pure bred hosts sampled from outside the zone (Sage *et al.*, 1986; Moulia *et al.*, 1991). A similar phenomenon has also been recorded for fish (Dupont & Crivelli, 1988). Alternatively, the work of Coustau *et al.*

(1991) and LeBrun *et al.* (1992) indicates that a particular parental host genome may be able to resist infection by specific parasites; therefore, the selective pressure may favour the non-susceptible genome (and their closely associated hybrids), displacing the competing (susceptible) species. By laboratory investigation, it has been demonstrated that hybrid mice have a genetic predisposition to infection by the nematode *Aspicularis tetraptera* (see Moulia *et al.*, 1993) and the cestode *Hymenolepis citelli* (see Wassom *et al.*, 1973, 1974, 1986; Munger *et al.*, 1989). Predisposition has also been proposed for mice infected by the nematode *Heligosomoides polygyrus* (see Scott, 1988), guppies infected by the monogenean, *Gyrodactylus bullatarudis* (see Scott, 1985) and mussels infected by the trematode *Prosorhynchus squamatus* (see Coustau *et al.*, 1991).

Within the genetic interactions which occur in zones of hybridisation (Barton & Hewitt, 1985; Hewitt, 1988), the selective pressure exerted by parasitic infection may, in some systems, reduce the fitness of the hybrids with respect to that of either parent (Sage *et al.*, 1986), although the significance of this has been questioned (Klein, 1988). Such a phenomenon would add another contributory factor to the selection against hybrids which typically occurs via morphological aberrations or sterility. Selection against hybrid genomes would favour the maintenance of clines by balancing the effects of random dispersal, resulting in a 'tension zone' (Barton & Hewitt, 1989). By sampling *Scaphiopus* and *Bombina* in areas of sympatry, the distribution of helminths within parental genomes and an array of recombinant genotypes can be assessed. In the *Hyla* system parasites will have been confronted with the instantaneous doubling of the hosts genome. This

not only allows the testing of host susceptibility but also parasite specificity. In the *Scaphiopus* and *Hyla* systems, it is conceivable that rather than a single species (*N.scaphiopodis* and *P.nearcticum*) infecting both hosts, there are separate cryptic, species-specific mongeneans. With direct reference to research concerning polystomatid monogeneans, Prudhoe & Bray (1982) commented on the two approaches taken by authors. Firstly, that of the comparative morphologist, with species being distinguished by variation in a number of taxonomic characters. The other approach is that of the experimentalist, using laboratory experiments to make assumptions on events occurring in nature. It was an aim of this study to make use of both approaches and a primary remit of this study was to test specificity, in the laboratory. This was addressed by reciprocal cross-infections from electrophoretically-typed recipients with larvae taken from pre-typed donors, to augment extensive morphological comparisons made from field collections.

The ecology of the host will have a profound influence on transmission opportunities for parasites, particularly those which employ aquatic infective stages, intermediate hosts or have only one opportunity to establish infection during the hosts life-cycle. Brandt (1936) and Campbell (1968) first noted that the diversity of amphibian parasites may be directly related to their ecology, with greater diversity in the most aquatic hosts. However, Campbell (1968) found that terrestrial species harboured the heavier burdens. In their review Prudhoe & Bray (1982) stated that '..it seems very likely that ecological influences affect supposed host-specificity of platyhelminths in amphibians more than any other factor' which has subsequently been supported by field data. LeBrun, Renaud & Lambert (1990)

reported that although certain members of the monogenean genus *Diplozoon* were species specific, the specificity of *D.gracile* was principally dependent on host ethology rather than the physiological demands of the parasite. Therefore, in addition to host genetics, host ecology is also to be considered as a possible factor in influencing the distribution of parasites.

As stated above, in the first quantitative assessment of parasite distribution within host populations, Crofton (1971) concluded that parasites were typically overdispersed, generally fitting a negative binomial distribution. Overdispersed distributions are those in which the variance is greater than the mean, with the minority of hosts harbouring the majority of parasites. The causal factors in producing aggregated distributions may include the viability, spatial aggregation and behaviour of infective stages, in addition to the potential for direct reproduction within the host. Host-mediated factors include their behaviour, diet, age and immunological capabilities.

Mathematical models and their relation to field data have provided a basis for testing the interactions within host-parasite systems (see Anderson & Gordon, 1982; Anderson & May 1978a, b; Crofton, 1971) and experimental studies have addressed specific questions regarding the overdispersion of parasites (Anderson, 1978; Anderson, Whitfield & Dobson, 1978; Anderson *et al.*, 1982; Keymer & Anderson, 1979). A further objective of this study was to assess the principal factors influencing the transmission dynamics of the *P.nearcticum-H.versicolor* system within controlled experimental infections.

There have been a number of studies concerning the post-oncomiracidial development of monogeneans, from both natural infections (Kearn, 1963; Llewellyn, 1960; Ogawa, 1984; Salami-Cadoux, 1975; Sproston, 1945; Thoney & Munroe, 1987; Wiskin, 1970) and laboratory investigations (Alvey, 1936; Frankland, 1955; Hoshina, 1968; Kasahara, 1967; Jackson & Tinsley, 1988; Tinsley & Owen, 1975). However, there is little information pertaining to the post-oncomiracidial development of anuran polystomatids, with only North American *N.scaphiopodis*/*P.americanus* (Tinsley & Earle, 1983; Tinsley & Jackson, 1986; Tocque, 1990) and the neotenic forms of three species of african *Polystoma* described (Kok, 1990; Kok & DuPreez, 1989; Van Niekerk, 1992). Therefore, in addition to detailed morphological analysis of adult worms, the pre-migratory development of *N.scaphiopodis* and the neotenic development of *P.nearcticum* was to be investigated.

This study involved fieldwork in the United States supported by laboratory investigations in London. Fieldwork consisted of a 7 week period in Arizona in 1991 and 4 weeks in 1992, examining *S.bombifrons* and *S.multiplicatus*. In addition, a 6 week period was spent at the University of Missouri for the studies on *H.chrysoscelis* and *H.versicolor*. Samples of *B.bombina* and *B.variegata* were collected from the former Yugoslavia by Dr. N. Sanderson and preserved in the field. The toads were dissected by the author at Q.M.W.

In summary, the major objectives of this study were to assess the influence of host genetic constitution on susceptibility to parasitic infection, with particular

reference to infection by larval and adult polystomatid monogeneans. The full helminthological investigations were also to be used to indicate the role of host ecology in the numbers and diversity of fauna recovered. In addition, the distribution of *P.nearcticum* infection was examined experimentally, by manipulating and controlling a number of factors.

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Chapter 2.

A re-assessment of the hybrid zone between *Scaphiopus bombifrons* and *S.multiplicatus* (Anura:Pelobatidae) in the San Simon valley, Arizona/New Mexico, U.S.A.

2.1 Abstract.

This chapter presents a re-assessment of the hybrid zone between the spadefoot toads, *Scaphiopus bombifrons* and *S.multiplicatus* in the San Simon valley, Arizona/New Mexico, U.S.A. For both field and laboratory studies, cellulose acetate gel electrophoresis of four independent diagnostic loci was applied. This technique facilitated the rapid and accurate typing of a total of 706 adult *S.bombifrons*, *S.multiplicatus* and their hybrids. In addition, an 8 character morphological index was undertaken, the mean scores for the three most common genotypes (pure-bred *S.bombifrons*, *S.multiplicatus* and F_1 hybrids) were significantly different (1-way ANOVA, $F_{2,445} = 6940.74$, $p < 0.001$). Pure parental forms did not overlap at the 95% confidence level. However, the majority of first generation hybrids and backcrosses fell within the ranges of parental species, confirming the need for electrophoretic typing. Furthermore, by measurement of the snout-vent length (SVL), *S.bombifrons* was found to be significantly larger than *S.multiplicatus* (Two sample t-test, $p < 0.001$).

In addition to pure *S.bombifrons* and *S.multiplicatus*, first generation (F_1) hybrids and backcrosses to both parental types were present in the study area. Therefore, it is evident that several generations of inter-specific mixing occur. Gross regional differences, concerning pure-bred toads, were also apparent. In larger samples ($n > 10$) where hybrids were recovered, their mean frequency was 7.0% (range 3.2-9.6%) in 1991 and 4.3% (range 1.1-5.6%) in 1992. This study provides data, collected over a three year period, on the largest sample of adult *S.bombifrons*, *S.multiplicatus* and their hybrids yet described. By comparison with previous

examinations of this hybrid zone, suggestions that introgression is primarily controlled by the prevailing weather, ecology and sexual behaviour of the hosts are supported.

2.2 Introduction.

Scaphiopus bombifrons and *S.multiplicatus* are members of the amphibian family Pelobatidae Bonaparte, 1850. The genus *Scaphiopus* Holbrook, 1836 (incorporating the sub-genus *Spea* Cope, 1866) contains the six nearctic pelobatid species ranging from the southern United States to southern Mexico (Duellman & Trueb, 1986). The sub-genus *Spea* consists of *S.bombifrons*, *S.hammondii*, *S.intermontanus* and *S.multiplicatus*. *S.intermontanus* is confined to the Great Basin and its associated mountain ranges and *S.hammondii* to western California and northern Baja California. However, within the ranges of *S.multiplicatus* and *S.bombifrons*, areas of sympatry occur in the southern Great Plains of Arizona, Colorado, New Mexico, Oklahoma, Texas and northern Mexico (Stebbins, 1985) (Fig 2.1).

Spadefoot toads possess 13 chromosomes (Duellman, 1967; Duellman & Trueb, 1986) and have been found to have low DNA content per cell (Goin, Goin & Bachmann, 1968). Such an attribute will be a major factor in allowing these anurans to colonise arid regions, where breeding typically occurs in ephemeral waters. This study was based in the San Simon valley of southeastern Arizona and southwestern New Mexico, an area of sympatry between *S.bombifrons* and *S.multiplicatus*. The vegetation is

Chihuahuan desertscrub (formed of creosote, mesquite and ocotillo) and desert grassland (Lowe, 1964). The altitude of the study area ranges from 1280m on the valley floor at Rodeo, New Mexico to 1520m at Portal, Arizona (Dimmitt & Ruibal, 1980). Approximately half of the average rainfall (225mm) falls in monsoonal rains during July to September (Ruibal, Tevis & Roig, 1969). These summer monsoons are created from warm, moist air, moving over the mountainous terrain, resulting in convection storms.

The inter-relationships of spadefoot toads have been investigated by analyses of immunology (Sage, Prager & Wake, 1982); enzyme electrophoresis (Sattler & Mecham, 1979; Sattler, 1980, 1985; Wiens & Titus, 1991), fossil data (Kluge, 1966; Zweifel, 1956), karyotype (Wasserman, 1970; Wasserman & Bogart, 1968), skeletal morphology (Tanner, 1989) and experimental crosses (see Brown, 1967; Wasserman, 1957, 1964, 1970, Wasserman & Bogart, 1968). Within the genus, morphology is conservative but *S.bombifrons* and *S.intermontanus* are distinguished by a frontoparietal boss. The boss is formed of dermal bone which appears as a raised ridge at the anterior of the interorbital region and provides a clear diagnostic feature. Although Sage *et al.* (1982), Sattler (1980, 1985) and Wiens & Titus (1991) have produced slightly different phylogenies, certain findings are consistent. Firstly, *S.multiplicatus* and *S.bombifrons* are the most distantly related members of the sub-genus *Spea*. Secondly, *S.hammondii* is more closely related to *S.bombifrons* than *S.multiplicatus*. This confirms the division of the morphologically similar *S.hammondii* and *S.multiplicatus* to separate species, originally based on differences in behaviour and ecology (Brown, 1976). Sage *et*

al. (1982) proposed that divergence within the sub-genus *Spea* may have begun as recently as 6 million years B.P. However, debate continues as Tanner (1989) proposed that there was sufficient morphological, ecological and life-history data to separate *Scaphiopus* and *Spea* at genus level and that 3 sub-species of *Spea hammondi* were present (*S.hammondi hammondi, S.h.stagnalis, S.h.multiplicata*).

Sattler (1978, 1980, 1985), Simovich (1985) and Simovich & Sassaman (1986) noted that, on the basis of electrophoretic markers, pure and hybrid offspring of *S.bombifrons* and *S.multiplicatus* could be identified with a high degree of accuracy. Previous studies had shown little variation in karyotype (Wasserman, 1970), with some variation in morphology (Hughes, 1965; Tanner, 1989); male mating-call (Blair, 1956; Brown, 1976; Forester, 1969, 1973; Pierce, 1976) and calling position (Bragg, 1945; Brown, 1976; Lowe, 1954; Sattler, 1978). In hybrid zones, morphology may not provide a reliable indicator of genotype (see Feder, 1979; Gartside, 1980; Maxson & Wilson, 1974; Platz, 1972). Simovich (1986) employed a eight point morphological index which was found to be a poor tool for identification of hybrids compared with isozyme electrophoresis.

The examination of *Scaphiopus spp.* isozymes by starch gel electrophoresis has been comprehensive (Sattler, 1980, 1985; Sattler & Mecham, 1979; Simovich, 1985, 1986). Sattler (1985) reported 7 polymorphic loci between *S.bombifrons* and *S.multiplicatus* - albumin (Alb), haemoglobin (Hb), isocitrate dehydrogenase (Idh-1 & Idh-2), lactate dehydrogenase (Ldh-1), malate dehydrogenase (Mdh-1), superoxidase dismutase (Sod-1). For field studies, Simovich (1985) found that by

use of four loci, (Idh-1; Idh-2; Ldh-1; Mdh-1), all F_1 hybrids and 87.5% of backcrosses could be identified. In the San Simon valley, Mdh-1 is characterised by a single slow band, expressed by *S.bombifrons*, a single fast band, expressed by *S.multiplicatus*, with heterozygotes being triple banded. Ldh-1 is characterised by a single fast band, expressed by *S.bombifrons*, and two slower forms, expressed by *S.multiplicatus*. The overall profile of lactate dehydrogenase fits a tetrameric pattern with homozygotes producing 5 bands, heterozygotes being multiple-banded, comparable to those recorded for *Bufo spp.* and *Rana spp.* (Dessaur, 1974; Salthe, 1969; Wright, 1975; Wright & Moyer, 1966, 1968; Wright & Subtelny, 1971). Idh-2 is also dimeric, with a single slow band characteristic of *S.multiplicatus*, a single faster band expressed by *S.bombifrons* with heterozygotes being triple banded. The interpretation of the Idh-1 region has been a matter of conjecture. Sattler & Mecham (1979) and Wiens & Titus (1991) considered this region to be the result of gene duplication of an ancestral Idh-1 locus. Simovich & Sassaman (1986) considered post-translational modification to be the causal factor of the patterns observed. Linkage analysis by Simovich & Sassaman (1986) revealed that the alleles coding for Idh-1, Idh-2, Ldh-1, and Mdh-1 exhibited Mendelian segregation at four independently assorting loci.

S.bombifrons is generally considered to be a plains species (Stebbins, 1985), inhabiting lower elevation grasslands and river valleys (Tanner, 1989; Wasserman, 1964). Indeed, Simovich (1985) recorded a significant negative correlation between elevation and frequency of *S.bombifrons*. *S.multiplicatus* favours the desert scrub at higher elevations, being less abundant in grassland areas (Ruibal *et*

al., 1969; Tanner, 1989). Male *S.bombifrons* exhibit two mating call types, a 'fast' call in the south and west and a 'slow' call in the north and eastern extent of their distribution. Pierce (1976) found that the 'fast' call differed most from that of male *S.multiplicatus* and it is this form that is found in the San Simon valley (Sattler, 1978; Pierce, 1976). However, Sattler (1980) found no electrophoretic difference between the two forms. *S.multiplicatus* is the more common species in the study area and it has been suggested that the irregular distribution of *S.bombifrons* is related to the soil preference of adults (Simovich, 1985).

The history of the initial contact between *S.bombifrons* and *S.multiplicatus* remains unclear, although at present their ranges overlap broadly (Fig.2.1). By crossing *S.hammondi*, *S.bombifrons* and *S.intermontanus*, Brown (1967) proposed that members of the genus *Spea* have a high degree of interfertility. A number of zones of hybridisation have been recorded, including the San Simon valley (Brown, 1976; Forester, 1973; Hughes, 1965; Sattler, 1978). Interspecific matings of pure *S.bombifrons* and *S.multiplicatus* produce infertile males and females are approximately 45% as fecund as pure genotypes (Simovich, 1985). In regions where the 'slow-call' form of *S.bombifrons* is sympatric with *S.multiplicatus*, F_1 males may be partially fertile (Brown, 1976; Forester, 1969, 1975; Littlejohn, 1959; Wasserman, 1970). In nature, several generations of interbreeding must have occurred as backcrosses are present (Sattler, 1978). Certain authors have suggested that ecological changes instigated by the activities of man may have been a causal factor in the breakdown of reproductive isolation between anurans, by restricting the ability of a species to express an ecological preference (Frost &

Berga, 1977; Gerhardt, Guttman & Karlin, 1980; Mecham, 1960). This hypothesis has also been applied to *Scaphiopus spp.* (Bragg, 1965; Simovich, 1985; Wasserman, 1957).

In areas where introgression occurs, the frequency of hybrid genotypes has been estimated at approximately 4%, on the basis of male mating call (Forester, 1969) and cranial morphology (Hughes, 1965). From electrophoretic examination, Sattler (1978) estimated that 5.7% of adult toads in the San Simon valley were from heterospecific crosses. In this region, the most common type of backcross results from male *S.multiplicatus* fertilizing the eggs of hybrid females (Simovich, 1985). Simovich (1985) found that the degree of hybridization varied both spatially (between ponds) and temporally (within ponds, between breedings). In addition, larger ponds contained fewer hybrid types than expected (based on the random assortment of marker alleles). However, by the same premise, matings at smaller ponds were more variable and approached random. Simovich (1985) concluded '...the extent of hybridization between *S.multiplicatus* and *S.bombifrons* and the distribution of pure and mixed genotypes are extremely heterogeneous on a fine scale'.

Due to the ephemeral nature of the desert ponds, spadefoot toads must exploit the summer monsoons at the earliest opportunity for successful breeding. The temporary ponds begin to fill on the first night of heavy rain and the toads emerge from their burrows to congregate in the pools (Bragg, 1965; Dimmitt & Ruibal, 1980; Forester, 1973; Ruibal *et al.*, 1969). With the heavy rains, the toads cease

hibernation and move directly to breeding ponds, rarely feeding en route (Ruibal *et al.*, 1969).

Forester (1969, 1975) showed that females respond positively to the calls of conspecific males. Furthermore, Woodward (1982) recorded non-random breeding for *S.multiplicatus* on the basis of snout-vent length (SVL). In the San Simon valley, Sattler (1978) noted that male *S.bombifrons* call from a stationary position on the edge of a pond, resulting in a ring of males around the periphery. In addition, their call is more divergent from *S.multiplicatus* than elsewhere in their range (Pierce, 1976). Male *S.multiplicatus* were found to be active searchers for mates, calling from floating positions. Theoretically, the direction of introgression should be biased towards the genome of *S.bombifrons*. However, in nature, Simovich (1985) found that due to the relative scarcity of *S.bombifrons* in this region, and the non-preferential behaviour of *S.multiplicatus* males, the majority of heterospecific matings involved the latter. This fits the 'common male:rare female' pattern described for other systems (see Avise & Saunders, 1984; Crenshaw, 1965). Simovich (1985) suggested that each species may have been responding to its own density, as Sattler (1985) found that in Texas, males of both species called from open areas within the pond. Variation in call position has also been noted in other regions (Bragg, 1945; Brown, 1976; Forester, 1969; Lowe, 1954; Sattler, 1978). Female choice may be negated by the overcrowding of breeding sites, leading to 'scramble competition' (Wells, 1977). Simovich (1985) found that isolating mechanisms were sufficient in large ponds, whereas in small or crowded ponds there was an increased frequency of mis-matches which may approach

random mating. As assortative mating is evident in large ponds, Simovich (1985) contested the suggestion by Sattler (1978) that the high level of hybridisation was based on the near equality of adult species, but instead due purely to small, crowded congresses.

Simovich (1985) stated that in syntopic ponds, the frequency of hybrid tadpoles typically ranged from 0 to 16%, although a maximum of 40% was recorded. A smaller scale examination of 4 ponds, found that the frequency of hybrid adults ranged from 2-31% (Simovich, 1985). By a repeated census at the same location it was also noted that the proportion of adult genotypes did not differ significantly between breedings in the same year. Simovich (1985) found that, within the local population of *S.multiplicatus*, the allele frequencies for Ldh exhibited a general pattern of homogeneity at each pond. This was suggested to indicate that the breeding ponds of the local population were not particularly discrete, inbred or subject to differing selection. Indeed, Simovich (1985) found that pure *S.multiplicatus* ponds were often in close proximity to hybrid ponds (although this may have been related to differences in elevation).

The longevity of ponds in the San Simon valley is directly related to the preceding rainfall, amount of run-off received, dimensions of the pool and prevailing weather conditions (Simovich, 1985). Tadpole mortality may be high as a result of drying and predation, leading to significant selection pressures for rapid development (Caldwell, Thorp & Jervey, 1980; Creuser & Whitford, 1976; Licht, 1974; Mayhew, 1965; Travis, 1983; Wilbur, 1977). Spadefoot toads develop

rapidly, for example, *S.bombifrons* takes just 20 hours to hatch at 30°C (Justus *et al.*, 1977 cited by Duellman & Trueb, 1986) and may reach metamorphosis in 13 to 15 days (King, 1960; Voss, 1961). However, differences between the species do occur, for example, Zweifel (1968, 1977) found that, at a constant temperature of 32°C, *S.bombifrons* tadpoles reached metamorphosis 8.5 days faster than *S.multiplicatus* (19.5 days and 28 days respectively). Brown (1967) recorded that *S.hammondi* possessed a rapid rate of development both in cold and warm-adapted populations and that disjunct populations possessed differing thermal tolerances related to the prevailing climate. Differences in developmental rate have also been documented between tadpoles of varying genotype, with hybrid crosses following a similar pattern to *S.bombifrons* (see Simovich, 1985). *S.bombifrons* and hybrids may also produce a higher percentage of carnivore morphs, which develop faster than herbivores (Pomeroy, 1981 cited by Simovich, 1985). Trade-offs between developmental rate and size at metamorphosis could possibly explain the differences observed, however, Simovich (1985) noted that the longer developmental time did not afford any significant size advantage to *S.multiplicatus*.

This dynamic hybrid zone in the San Simon valley has previously been examined by Sattler (1980, 1985) and Simovich (1985). In this study, it was hoped to provide a wider sample of adult genotypes in order to assess the success of interspecific crosses. In addition, comparisons of the electrophoretic profile could be made using a different separation medium, cellulose acetate. Morphological examination was also employed to check the consistency of previous findings (Simovich, 1985; Simovich & Sassaman, 1986) upon a larger sample.

2.3 Materials and Methods.

2.3.1 Collection of Spadefoot toads.

Fieldwork was based at the Southwestern Research Station (S.W.R.S.) of the American Museum of Natural History, Portal, Arizona. Collections of adult *S.bombifrons*, *S.multiplicatus* and their hybrids were made in the summers of 1990, 1991 and 1992. In 1990, a single sample of 110 toads was combined from 3 locations, Portal Road, State Line Road and Sulphur Draw (see below for locations). In 1991, samples totalling 341 individuals were collected from 16 sites and, in 1992, 6 sites were sampled (5 repeated from 1991), totalling 255 specimens. In all, 706 adult spadefoot toads were genotyped from the 3 years of sampling. The collection sites are mapped in Fig.2.2. and summarised below, with sample size per site in brackets.

1. Cochise Cactus Road, pond next to road, 0.3km W Hwy 80, 2km N jct Hwy 9, (n = 3 in 1991).

2. Animas Road (Hwy 9), roadside pond, 2km E jct of Hwy 80, (n = 3 in 1991).

3. Bridge on Route 80, bridge culvert, 0.5km S jct Hwy 80 & Hwy 9, (n = 5 in 1991).

4. Dancers Tank, cattle tank, 0.05km W of San Simon Road, 1km N jct Portal Rd, (n = 35 in 1991).

5. Peach Orchard Road, 2 cattle tanks, 0.5km S of road, 1km NE jct Portal Rd, (n = 14 in 1991).

6. Portal Road, roadside collection, pooled from Portal to jct State Line Rd, (n = 94 in 1991; n = 18 in 1992).

7. Next to Windmill Pond, small pond at bottom of cattle tank wall, 0.2km NE of Portal Rd, 1km NW jct of State Line Rd, (n = 12 in 1991).

8. Number 1 Pond, small pond, 0.1km S of Painted Mountain Road, 0.4km SW jct Portal Rd, (n = 14 in 1991).

9. Painted Mountain Road, cattle tank 0.15km S of road, 0.5km SW jct Portal Rd, (n = 2 in 1991).

10. Millers Pond, Millers Ranch, cattle tank, 0.1km jct Portal Rd, (n = 82 in 1991; n = 91 in 1992).

11. North of Rodeo (Hwy 80), roadside collection, pooled between jct Hwy 9 and Rodeo, (n = 2 in 1991).

12. Luthers Field, flooded field, 0.1km W State Line Road, 1.5km N jct Hwy 80, (n = 5 in 1991).

13. State Line Road, roadside collection, pooled between Portal Road and 0.5km N jct Hwy 80, (n = 10 in 1991; n = 4 in 1992).

14. Black Dog Pond, cattle tank, 0.2km W of Sulphur Draw Road, 3km jct of State Line Road, (n = 31 in 1991).

15. Sulphur Draw, cattle tank and roadside collection, 0.1-0.3km jct of State Line Road, 0.1km N Hwy 80, (n = 19 in 1991; n = 40 in 1992).

16. South of Rodeo (Hwy 80), roadside collection, pooled between Rodeo and 5km S on Hwy 80, (n = 10 in 1991; n = 10 in 1992).

17. Skeleton Canyon Road, cattle tank and roadside collection, 0.1-2km E jct Hwy 80 and Apache, 15km S of Rodeo (n = 92 in 1992).

2.3.2 Morphological Analysis.

Prior to typing, the SVL of each toad was measured with vernier callipers to the nearest mm. Individuals were then assessed for 8 characters to produce a morphological index. These attributes were:

1. inter-orbital boss height
2. lateral profile of nose
3. contrast and extent of the inner pair of dorsal stripes
4. contrast and extent of the outer pair of dorsal stripes
5. contrast and extent of head stripes
6. dark hourglass pattern on the head
7. colour of pigmented tubercles
8. proportion of pigmented tubercles (other than white or yellow)

These characters were based on Simovich (1985), Hughes (1965) and Stebbins (1985). For each of the above, a score of 1 was recorded for a *S.bombifrons* character, 2 for intermediate and 3 for *S.multiplicatus* (refer to Table 2.1). The total score for each animal was noted, with a possible range of 8 to 24 points.

2.3.3 Electrophoresis.

Titan III (76x76mm) cellulose acetate gels were run in an Helena Zip Zone chamber (Cat. No. 1283). Samples were applied to the gel using a Helena Super Z-12 kit (Cat. No. 4084 & 4085) and an perspex aligning base made by the author. Electrophoresis was undertaken using a Biomed E250 power pack and, in Arizona, connected via a Hi-Fi quality transformer. The following chemicals were

supplied by Sigma, Poole, Dorset; DL-Isocitric acid (I-1252), DL-Lactic Acid (L-1375), L-Malic acid (M-1000), NAD (N-7381), NADP (N-0505), $MgCl_2$ (M-8266), MTT (M-2128), PMS (P-9625), Trizma Base (T-1503), Glycine (G-7126) and 2-Phenoxyethanol (P-1126). Hydrochloric acid (BDH-10307 ANALAR) was supplied by BDH Chemicals, Poole, Dorset.

2.3.3.1 **Preparation of Gel, Electrode and Stain Buffers.**

a) Tris-Glycine Buffer. 30g Trizma base
144g Glycine

Made up to 1 litre and diluted 1:9 with distilled water for general use.

b) Tris-HCl pH=7. 11.1g Trizma base
87.5ml 1M HCl

c) Tris-HCl pH=8. 11.1g Trizma base
62ml 1M HCl

d) Tris-HCl pH=9. 24.65g Trizma base
30ml 1M HCl

Made up to 1 litre, pH adjusted dropwise with HCl.

2.3.3.2 **Preparation of Stock Stain Recipes.**

Basic stock solutions were made up in the following concentrations, using distilled water:

NAD	:	[2mg/ml]
NADP	:	[2mg/ml]
MTT	:	[10mg/ml]
PMS	:	[2mg/ml]
$MgCl_2$	:	[20mg/ml]
DL-Isocitric acid	:	[100mg/ml]

2.3.3.3 **Preparation of Stain Recipes.**

a) Isocitric dehydrogenase (Idh) : **EC 1.1.1.42***
1.0ml Tris-HCl, pH = 7.0
1.5ml of NADP
15 drops of DL-Isocitric acid
8 drops of $MgCl_2$
5 drops of MTT
5 drops of PMS.

b) Lactate dehydrogenase (Ldh) : **EC 1.1.1.27**
1.0ml Tris-HCl, pH = 7.0
1.5ml of NADP
10 drops of DL-Lactic acid
5 drops of MTT
5 drops of PMS

c) Malate dehydrogenase (Mdh) : **EC 1.1.1.37**
1.0ml Tris-HCl, pH = 8.0
1.5ml of NAD
13 drops of Malic substrate
5 drops of MTT
5 drops of PMS

Malic substrate : 180ml of distilled water
20ml Tris-HCl, pH = 9.0 } pH adjusted with HCl
3.68g of L-Malic acid

(* : reference number according to the Enzyme Commission, 1973)

2.3.3.4 **Sample Preparation.**

The two distal phalanges of digit IV from the right foot of each toad were removed with sterile surgical scissors (nomenclature following Duellman & Trueb, 1986). The foot was liberally dusted with Veterinary Wound Powder (Battle, Hayward & Bower Ltd, Lincoln). For each run, 10 individuals were sampled. Each toe was placed in a marked eppendorf tube containing 1 drop of 0.6% boiled saline and kept on ice. The skin and muscle were removed from the bone with the aid of a dissection microscope, cold-light source, scalpel, seeker and chilled watch-glasses.

Immediately prior to electrophoresis, tissue was homogenised on ice using a ground glass homogeniser. A grinding medium of equal quantities of stock NAD, NADP (enzyme co-factors) and a 2% 2-phenoxyethanol solution (liberator of membrane bound proteins) was used. Homogenisation continued for one minute, the sample returned to its respective eppendorf and the homogeniser washed vigorously with 3 changes of chilled distilled water.

2.3.3.5 **Preparation of Equipment and Consumables.**

The chamber was stored in a fridge prior to a run, which reduced buffer evaporation, restricted fungal growth and ensured a temperature of 2 to 4^0C. Buffer wells contained wicks supplied by Helena and were filled with a total of 100mls of gel buffer. The chamber was cleaned regularly, with the buffer and wicks replaced. The enzyme system and sample order were permanently marked on the mylar side of the gel. Each gel was then soaked for at least 20 minutes in gel buffer. The applicator kit was chilled prior to use and primed following manufacturers instructions. An 8µl aliquot of supernatant homogenate was sufficient for 3 gels. The excess moisture on each gel was removed by the gentle application of filter paper before positioning the gel in the aligning base. For exact and repeatable loading, the base was precisely constructed to prevent any freedom of movement. For Mdh & Ldh gels, 4 applications were made per load zone, 6 for Idh. The gels were run for 15 minutes at a potential difference of 200 volts.

Stains were made fresh and kept in blacked-out vials to protect the photosensitive PMS and MTT. After electrophoresis the gels were placed, acetate side up, in a

plastic tray. The stain mixture, having been mixed vigorously, was poured onto 2 layers of Whatman No.1 filter paper cut to 76x76mm (adapted from Thompson & Davies, 1989). The stain was applied to the gel, and the tray covered for incubation in an oven at 37°C. On average, incubation times ranged between 5 minutes for Mdh to 15 minutes for Idh. The gels were scored after rinsing in tap water then dried overnight for permanent storage. Hybrids were checked by the use of line-up gels.

2.3.3.6 Summary of Conditions.

Plate	Titan III Cellulose Acetate (76x76mm).
Soaking time	20 minutes.
Sample volume	8µl for 3 gels.
Chamber voltage	200 volts.
Electrophoresis time	15 minutes.
Stain volume	2ml.
Incubation temperature	37°C.
Stain time	5 to 15 minutes.

2.3.3.7 Definition of genotype.

1. *S.bombifrons*: *S.bombifrons* genotype at all 4 diagnostic loci.

2. *S.multiplicatus*: *S.multiplicatus* genotype at all 4 diagnostic loci.

3. F_1 hybrid: heterozygous for *S.bombifrons* and *S.multiplicatus* alleles at all 4 diagnostic loci.

4. Backcross to *S.bombifrons*: homozygous for *S.bombifrons* alleles at some loci, heterozygous at the reminder.

5. Backcross to *S.multiplicatus*: homozygous for *S.multiplicatus* alleles at some loci, heterozygous at the remainder.

6. Double backcross: homozygous for both *S.bombifrons* and *S.multiplicatus* alleles at some loci, heterozygous at the reminder.

The final category can be classified as a double backcross, as all F_1 males in this region are thought to be sterile (Simovich, 1985).

2.4 Results.

The morphological index correctly identified pure parental types (Fig.2.3A) and the scores for the three most common genotypes (*S.bombifrons*, *S.multiplicatus* and F_1 hybrids) were significantly different (1-way ANOVA, $F_{2,445} = 6940.74$, $p < 0.001$). Pure parental types did not overlap at the 95% confidence level (Fig.2.3B). However, only 20% of all hybrid genotypes were identified by morphology alone (23% of F_1 hybrids, 16.7% of backcrosses to *S.multiplicatus* and 0% of backcrosses to *S.bombifrons*) also a number of hybrids exhibited the parental extreme for all 8 characters.

For pooled samples of pure species, male to female ratios were 2.4:1 for *S.bombifrons* and 2:1 *S.multiplicatus*. Snout-vent length for each species is plotted in Fig.2.4. For statistical comparison sexually immature toads (below 35mm SVL) were omitted, and *S.bombifrons* was found to be significantly larger than *S.multiplicatus* (Two sample t-test, $p < 0.001$).

Although the four diagnostic loci were readily scored, with regard to sub-banding, the overall electromorphic patterns were different to those previously

described (Sattler, 1980, 1985; Sattler & Mecham, 1979; Simovich, 1985, 1986). Within pure-bred *S.multiplicatus*, consistent sub-banding was noted. As stated in the Introduction, *S.multiplicatus* exhibits two slower forms of Ldh-1 (than *S.bombifrons*) plus a heterozygote. These three *S.multiplicatus* electromorphs can be linked to the other diagnostic enzymes. Individuals exhibiting the faster Ldh-1 band also possess a faint sub-band on the anodal side of Mdh-1, whereas the slow and heterozygotic forms did not. In addition, between the Idh-1 and Idh-2 bands individuals expressing the fast-Ldh form produce two sub-bands, the heterozygote produces one and the slow form none. The patterns for Idh-1 confirmed the reversal of descriptions by Sattler & Mecham (1979) proposed by Simovich (1985) and Simovich & Sassaman (1986).

The distribution of adult genotypes in the San Simon valley is recorded in Table 2.2 and Figs.2.5 & 2.6. The study area contains both *S.bombifrons* and *S.multiplicatus*, in addition to first generation (F_1) hybrids and backcrosses to both parental types, although no double backcrosses were recorded. It is evident that several generations of inter-specific mixing were present in this region. Gross regional differences were also apparent, with *S.multiplicatus* being the most frequent and widespread of the species (frequencies ranging from 25-100%). *S.bombifrons* was found only at the southern end of the study area at frequencies ranging from 0-70%. This species also appears to follow the topography of the valley, being restricted to the lower elevations (the valley floor approximates to the line of Highway 80 and the State Line Road and rises on either side).

In larger samples (n > 10) where hybrids were recovered, the proportion of hybrid individuals was 7.0% (range 3.2-9.6%) in 1991 and 4.3% (range 1.1-5.6%) in 1992. At the two sites where the proportion of hybrids exceeded the ranges noted above, both involved small samples (site 9, 1991, n = 2; site 13, 1992, n = 4). Hybrid offspring, possessing *S.bombifrons* alleles were occasionally found at sites where the pure parental type was not recovered (site 5, 9, 1991; site 6, 1992). However, it should be noted that sample sizes did not exceed 20 and pure *S.bombifrons* was found in close proximity to these sites. Although slightly underestimated by the use of 4 diagnostic loci, backcrosses were found at frequencies of 1.2-6.4% (pooled for backcrosses to both parental types). Only a single backcross to *S.bombifrons* was recorded (from the southern end of the study area), the remaining 11 were to *S.multiplicatus*. At sites where sample sizes exceeded 10 and the less common *S.bombifrons* was present in higher proportions (producing a more even distribution of the parental types), there were not necessarily higher frequencies of hybrid genotypes.

The pooled samples for the three subsequent years (Table 2.2) show a wide variation in species composition. However, by the nature of the prevailing weather conditions, breeding did not occur at all sites in a particular year, restricting opportunities for sampling. Therefore, statistical analysis centred on genotypes recorded at localities sampled in both 1991 and 1992 (sites 6, 10, 13, 15, 16) and was evaluated by a GLIM test (assuming poisson error). The results are presented in Table 2.3. The first 4 mean deviance ratios indicate that there were significant differences in the mean number of toads between years, ponds, genotypes and

between the same pond in different years, all of which may have been influenced by sampling effort. However, having taken these factors into account, the final two mean deviance ratios indicate that the various genotypes were still found at significantly different frequencies at these different localities and the frequencies changed between years. It is also interesting to note (from the list of estimates) that the main factor in producing the differences between sampling years was the relative decrease in *S.multiplicatus*.

2.5 Discussion.

Cellulose acetate gel electrophoresis proved to be a rapid, accurate separation medium which, unlike acrylamide and starch, requires only a small amount of tissue homogenate. Three gels were run together allowing all loci to be scored for 10 individuals under identical conditions (although up to 20 would be possible). The exceptionally short run times (15 minutes) compared to 2-3 hours for other media, not only shortened the typing process but also reduced problems with fluctuations in the power supply (occasionally interrupted by storms during laboratory work at S.W.R.S.). Moreover, the removal of a minimum of tissue may also lead to an improved survival rate in studies where animals are re-released back to the wild. In this study, the advantages of cellulose acetate were in agreement with those proposed by Easteal & Boussy (1987).

The differences in banding patterns between this study and previous work (Sattler, 1980, 1985; Simovich, 1985; Simovich & Sassaman, 1986) is most probably due to the separation medium chosen. Starch gels separate proteins on

the basis of charge and molecular size, however, the large pores within the cellulose acetate matrix result in separation being primarily based on charge. The basis of the unique sub-banding within pure-bred *S.multiplicatus* is unknown, as the sub-banding is consistent, this suggests inherent genetic variability. However, post-translational factors may also be involved and further examination is required. Sattler & Mecham (1979) proposed that the banding and sub-banding of the Idh-1 region was the result of gene duplication, which was supported by Wiens & Titus (1991). Simovich (1985) and Simovich & Sassaman (1986) regarded this region to be due to post-translational modification. Although the findings of this study confirmed the reversal of descriptions by Sattler & Mecham (1979) proposed by Simovich (1985) and Simovich & Sassaman (1986), differences in motility were not noted, which supports the original hypothesis.

The morphological index proved to be an accurate tool in the discrimination of pure-bred adults, however, the majority of first generation hybrids and backcrosses fell within the ranges of parental species. Although a small proportion of backcrosses (12.5%) will also avoid detection by the enzyme systems chosen in this study, morphological variation underlines the significance of electrophoretic typing in areas of hybridisation. There was considerable agreement in the distribution of morphological scores with Simovich (1985), although the latter examined preserved (frozen) specimens. The only notable deviation regarded F_1 hybrids which, in this study, were not as readily distinguished from pure genotypes. For future examination, the morphology of the eye may be useful, with regard to pupil shape, iris pattern and colour. The significant difference in SVL

between the two species, would be difficult to apply to a morphological index on an individual basis.

In this study, the frequency of adult hybrids ranged from 2.7-6.0% (pooled samples) over 3 consecutive years. These findings are comparable to those of previous investigations (Forester, 1969; Hughes, 1965; Sattler, 1985). In addition, the gross distribution of genotypes is similar to those recorded for larval stages (Simovich, 1985 : see Fig.2.7). In the San Simon valley, the highest recorded frequencies of adult hybrids (31%) was considered to be due to the extremely heterogeneous nature of this hybrid zone (Simovich, 1985). Sites 5, 10, 14 & 17 were close to the 4 ponds sampled by Simovich (1985) but hybrid genotypes did not exceed 9.6%. In this study, although the number of locations sampled was four times greater, the possibility remains that sites of high hybrid frequency may have been missed. Alternatively, as variation also occurs temporally (indicated by the significantly different composition of genotypes from year to year), in recent years the dynamic nature of the zone may have resulted in fewer hybrids being produced. However, it should be noted that the closely related *S.couchii* may have a maximum life-span of 13 years (Tinsley & Tocque, 1994), thus it is possible that breeding congregations sampled in one year may reflect the dynamics of the zone over the previous decade.

As noted in the Introduction, the restricted distribution of *S.bombifrons* in this region may be related to soil preference (Simovich, 1985; Wasserman, 1957) and altitude (Simovich, 1985). In this study, the numbers of *S.bombifrons* recovered

from the same location varied significantly over two consecutive years, which may indicate the fluctuations of a more transient influence in distribution. The greater abundance of *S.bombifrons* at the southern end of the valley does not represent a simple cline of replacement from *S.multiplicatus*, as Pomeroy (1981) cited by Simovich (1985) recovered *S.multiplicatus* from the south of the valley. However, it should be noted that the samples of Pomeroy (1981) were taken at higher elevations.

Avise & Saunders (1984) reported that hybrid crosses of sunfish typically involved males of a more common species and females of a rarer species. The indiscriminate nature of mate choice by *S.multiplicatus* males and rarity of *S.bombifrons* females produces a similar effect in the San Simon valley (Simovich, 1985). In addition, in this study, the male:female ratio was found to be higher for *S.bombifrons* than *S.multiplicatus*. The abundance/non-preferential behaviour of male *S.multiplicatus* was supported by the excess of backcrosses to this species. These factors may have produced selection on the mating system. This is indicated by their call being more divergent from *S.multiplicatus* than in other areas of sympatry (Pierce, 1976) and by their preference to call from the periphery of ponds. The breakdown of isolating mechanisms at crowded ponds (Simovich, 1985) will play a significant role in the maintenance of the hybrid zone. In this study, where sample sizes exceeded 10, higher frequencies of hybrids were not necessarily found when the frequencies of parental types were more even. This finding concurs with Simovich (1985) and opposes Sattler (1978).

Post-mating selection will also have a major influence on the distribution of genotypes. Small and shallow ponds which lead to a high degree of hybridization (Simovich, 1985) are also the most ephemeral. Therefore, this will provide a selective advantage for *S.bombifrons* and hybrid tadpoles which have a faster developmental rate, earlier metamorphosis and higher frequency of carnivore morphs than *S.multiplicatus* (Pomeroy, 1981; Simovich, 1985). Such an advantage may help balance the disadvantageous pressures of infertility and reduced fecundity. The absence of an apparent trade-off between developmental rate and size at metamorphosis (Simovich, 1985) is of interest. Rafinska (1991) found that *Bombina variegata* lays smaller clutches of larger eggs than *B.bombina* (see Chapter 10). The tadpoles develop at the same rate as *B.bombina* but metamorphose, on average, 10 days earlier, which the author proposed was an adaptation to the more ephemeral nature of their primary choice of habitat. A comparable examination of *S.bombifrons* and *S.multiplicatus* may also provide some information as to the type of initial reproductive investment made by these toads. Simovich (1985) suggested that the cattle industry has increased the number of ephemeral ponds, thus encouraging hybrid success. However, the majority of cattle tanks are large and therefore more persistent, which should favour the tadpoles of *S.multiplicatus*.

Simovich (1985) concluded that, in the San Simon valley, introgression is highly variable with selective forces interacting to different degrees in each year/area. The prevailing weather conditions, ecology and sexual behaviour of the toads will have a major influence on the frequencies of genotypes observed. This study has

provided the most extensive assessment of adult genotypes yet described and the genotypic frequencies are comparable to those previously documented, indicating that this may indeed be a region of 'balanced introgression' of the type described by Forester (1975).

2.6 References.

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Character	Score		
	1	2	3
1	high	vague	none
2	sharp	vague	none
3	clear	vague	very vague/ none
4	clear	vague	very vague/ none
5	clear	vague	none
6	clear	vague	none
7	white/ yellow	lt. red/ orange	red
8	few	~1/2	>1/2

Table 2.1 Morphological index criteria (reference numbers as in the Method), a score of 1 was recorded for *S.bombifrons*, 2 for intermediate and 3 for a *S.multiplicatus* character (from Simovich, 1985).

A.

Site	*S.b.*	BKB	F_1	BKM	*S.m.*	n
Pooled	55.5	0	0	2.7	41.8	110

B.

Site	*S.b.*	BKB	F_1	BKM	*S.m.*	n
1	0	0	0	0	100	3
2	0	0	0	0	100	3
3	0	0	0	0	100	5
4	0	0	0	0	100	35
5	0	0	7.1	0	92.9	14
6	2.1	0	3.2	0	94.7	94
7	25.0	0	8.3	0	66.7	12
8	42.9	0	7.1	0	50.0	14
9	0	0	50.0	0	50.0	2
10	11.0	0	6.1	2.4	80.5	82
11	0	0	0	0	100	2
12	40.0	0	0	0	60.0	5
13	30.0	0	0	0	70.0	10
14	19.4	3.2	3.2	3.2	71.0	31
15	36.8	0	0	0	63.2	19
16	50.0	0	0	0	50.0	10
Pooled	12.6	0.3	3.8	0.9	82.4	341

C.

Site	*S.b.*	BKB	F_1	BKM	*S.m.*	n
6	0	0	5.6	0	94.4	18
10	42.9	0	1.1	4.4	51.6	91
13	25.0	0	25.0	0	50.0	4
15	45.0	0	2.5	2.5	50.0	40
16	70.0	0	0	0	25.0	10
17	33.7	0	1.1	0	65.2	92
Pooled	37.6	0	2.0	2.0	58.4	255

Table 2.2 Sample composition of *S.bombifrons*, *S.multiplicatus* and their hybrids collected in A.) 1990, B.) 1991 and C.) 1992 by percentage of the total (sites as described in the Method). Abbreviations: n = number of individuals sampled, *S.b.* = *S.bombifrons*, *S.m.* = *S.multiplicatus*, F = F hybrids, BKB = backcrosses to *S.bombifrons*, BKM = backcrosses to *S.multiplicatus*.

Regression analysis: Accumulated analysis of deviance.

Change	d.f.	deviance	Mean deviance	Mean deviance ratio
year	1	7.1762	7.1762	8.22*
pool	4	244.8100	61.2025	70.09*
genotype	4	634.5758	158.6440	181.67*
pool.year	4	60.1036	15.0259	17.21*
genotype.pool	16	79.1414	4.9463	5.66*
genotype.year	4	19.2080	4.8020	5.50*
residual	16	13.9720	0.8732	
total	49	1058.9870	21.6120	

Estimates of regression coefficients.

	estimate	S.E.	t
Constant	+0.214	+0.722	+0.30
year 1992	-0.487	+0.381	-1.28
pond 10	+2.381	+0.739	+3.22
pond 13	+0.536	+0.918	+0.58
pond 15	+1.319	+0.780	+1.69
pond 16	+1.314	+0.823	+1.60
genotype BKB	-9.4	+53.6	-0.18
genotype F	+1.004	+0.880	+1.14
genotype BKM	-9.6	+53.5	-0.18
genotype *S.m.*	+4.279	+0.724	+5.91
pond 10. year 1992	+1.436	+0.310	+4.63
pond 13. year 1992	+0.370	+0.673	+0.55
pond 15. year 1992	+1.968	+0.396	+4.97
pond 16. year 1992	+0.958	+0.559	+1.71
genotype BKB. pond 10	-3.1	+76.6	-0.04
genotype BKB. pond 13	-0.7	+74.7	-0.01
genotype BKB. pond 15	-2.3	+76.6	-0.03
genotype BKB. pond 16	-1.8	+76.0	-0.02
genotype F_1. pond 10	-2.377	+0.991	-2.40
genotype F_1. pond 13	-1.99	+1.43	-1.40
genotype F_1. pond 15	-3.37	+1.37	-2.46
genotype F_1. pond 16	-11.9	+53.9	-0.22
genotype BKM. pond 10	+7.6	+53.5	+0.14
genotype BKM. pond 13	-0.7	+76.4	-0.01
genotype BKM. pond 15	+6.5	+53.5	-0.12
genotype BKM. pond 16	-1.8	+76.0	-0.02
genotype *S.m.* pond 10	-2.724	+0.742	-3.67
genotype *S.m.* pond 13	-3.070	+0.948	-3.24
genotype *S.m.* pond 15	-3.192	+0.775	-4.12
genotype *S.m.* pond 16	-4.123	+0.860	-4.79
genotype BKB. year 1992	-0.5	+50.5	-0.01
genotype F . year 1992	-1.213	+0.694	-1.75
genotype BKM. year 1992	-0.101	+0.878	-0.11
genotype *S.m.* year 1992	-1.195	+0.290	-4.12

Table 2.3 GLIM analysis (assuming poisson error) for ponds 6, 10, 13, 15 & 16 in 1991 and 1992.

Footnote.
The estimates were derived from log. (# toads). Each sample was compared to the value fitted to pond 6, genotype *S.b.* in 1991. Comparisons resulting in values of $t > 2$ have a strong influence on the analysis. The values for the mean deviance ratio were compared to tabulated values of F, with numerator (change) d f. residual (16) d.f. * = significant at or above the 95% confidence level.

2.7 Legends.

Fig.2.1 Range map of *S.bombifrons* and *S.multiplicatus*, with areas of sympatry indicated by the overlapping ranges. Adapted from Sattler (1985) and Stebbins (1985).

Fig.2.2 The distribution of sampling sites of *S.bombifrons*, *S.multiplicatus* and their hybrids in the San Simon valley, Arizona/New Mexico, U.S.A. (sites are numbered as described in the text). Site 17 is not indicated. Stippled area indicates the extent of the Chiricahua mountain range. The valley floor approximates to the line of Highway 80 and the State Line road, rising on either side.

Fig.2.3 Correspondence between the morphological index and electrophoretic typing of adult *S.bombifrons*, *S.multiplicatus* and their hybrids. **A.)** frequency distribution of combined scores for all genotypes and **B.)** mean scores and 95% confidence limits for pure *S.bombifrons*, *S.multiplicatus* and F_1 hybrids.

Fig.2.4 Frequency distribution of host snout-vent length (SVL) of pooled samples of **A.)** *S.bombifrons* and **B.)** *S.multiplicatus* collected from 1990-1992.

Fig.2.5 Pie diagrams of the frequency of adult genotypes at each site in 1991. Genotypes indicated as follows: *S.bombifrons* = black, *S.multiplicatus* = white, F_1 hybrids = [] and pooled backcrosses = ▮.

Fig.2.6 Pie diagrams of the frequency of adult genotypes at each site in 1992. Genotypes indicated as in Fig.2.5. Site 17 displaced north solely for graphical comparison (refer to Materials & Methods for exact location).

Fig.2.7 Pie diagrams of the frequency of tadpole genotypes at 21 ponds in 1982 from Simovich (1985). Genotypes indicated as in Fig.2.5.

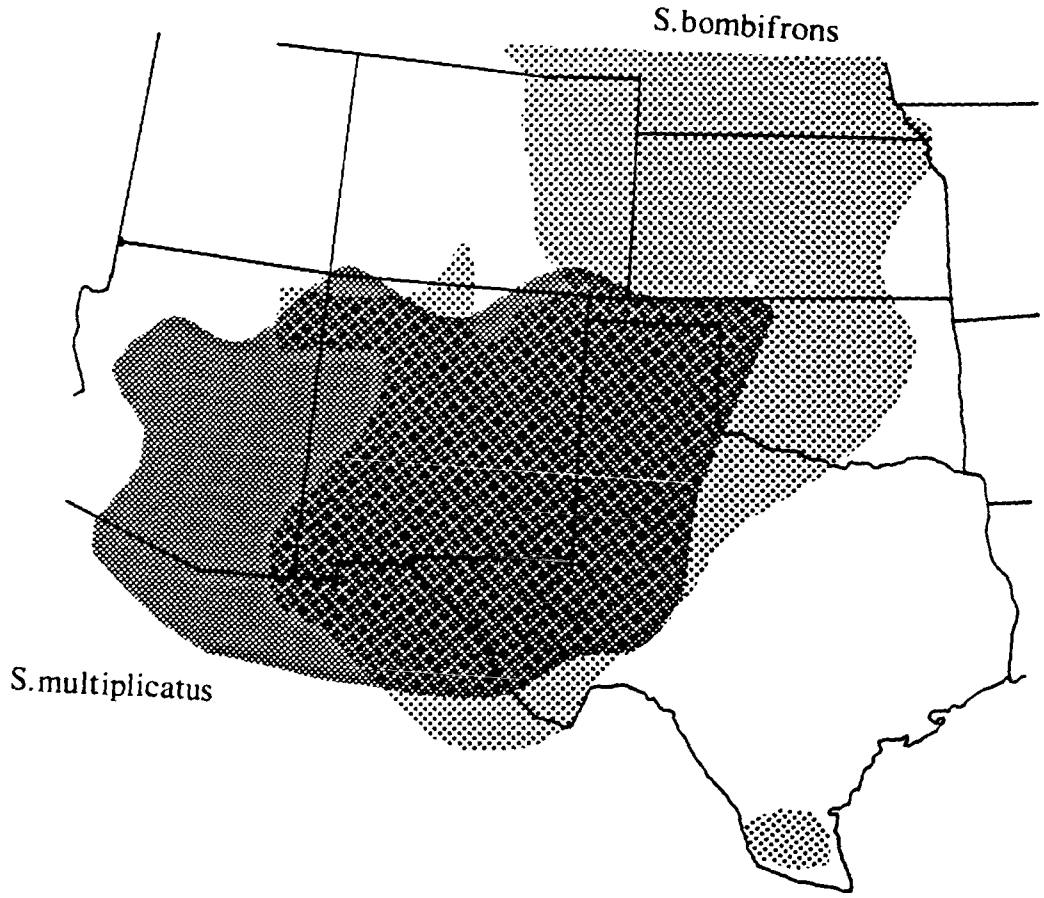


Fig.2.1

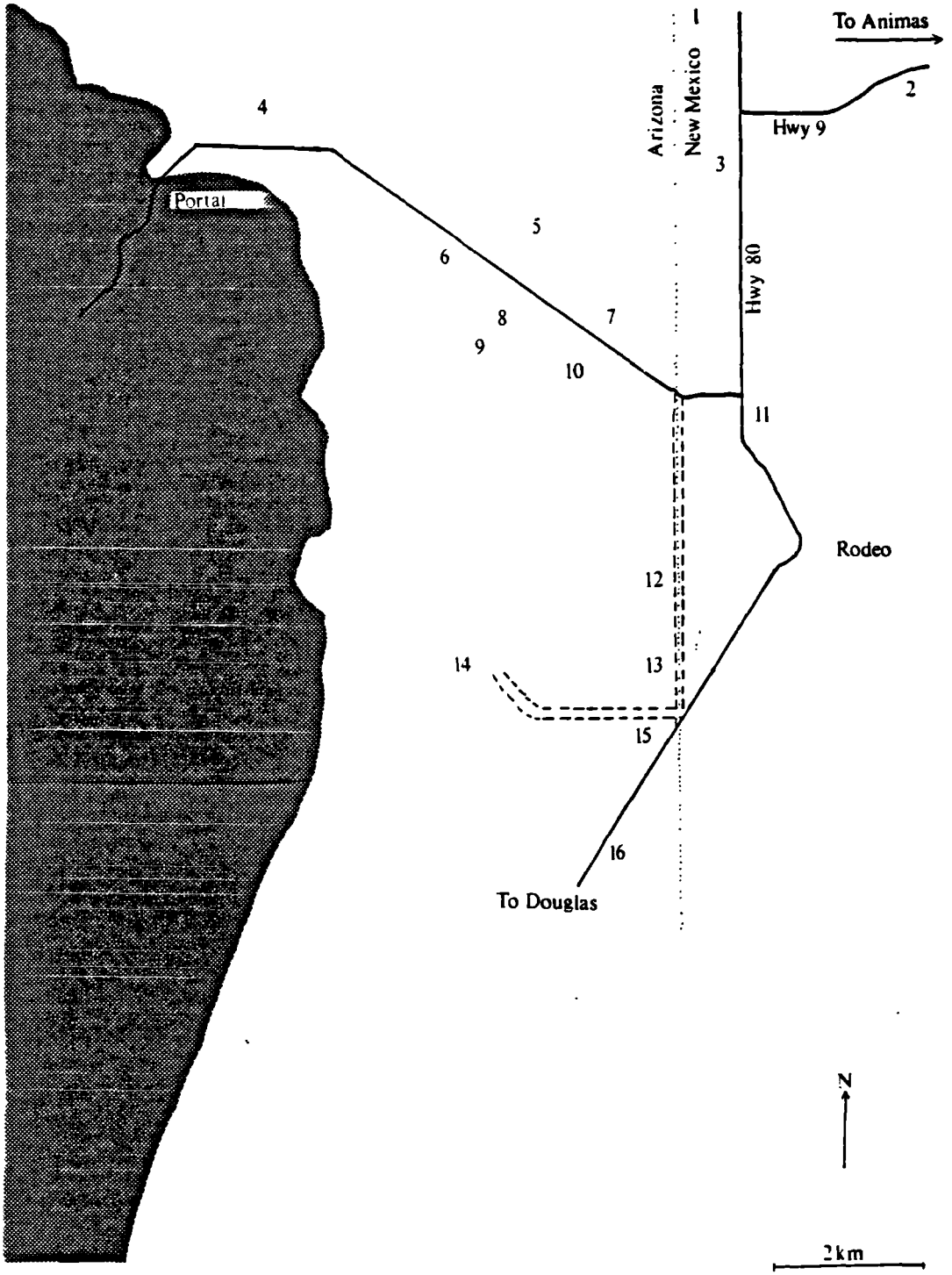
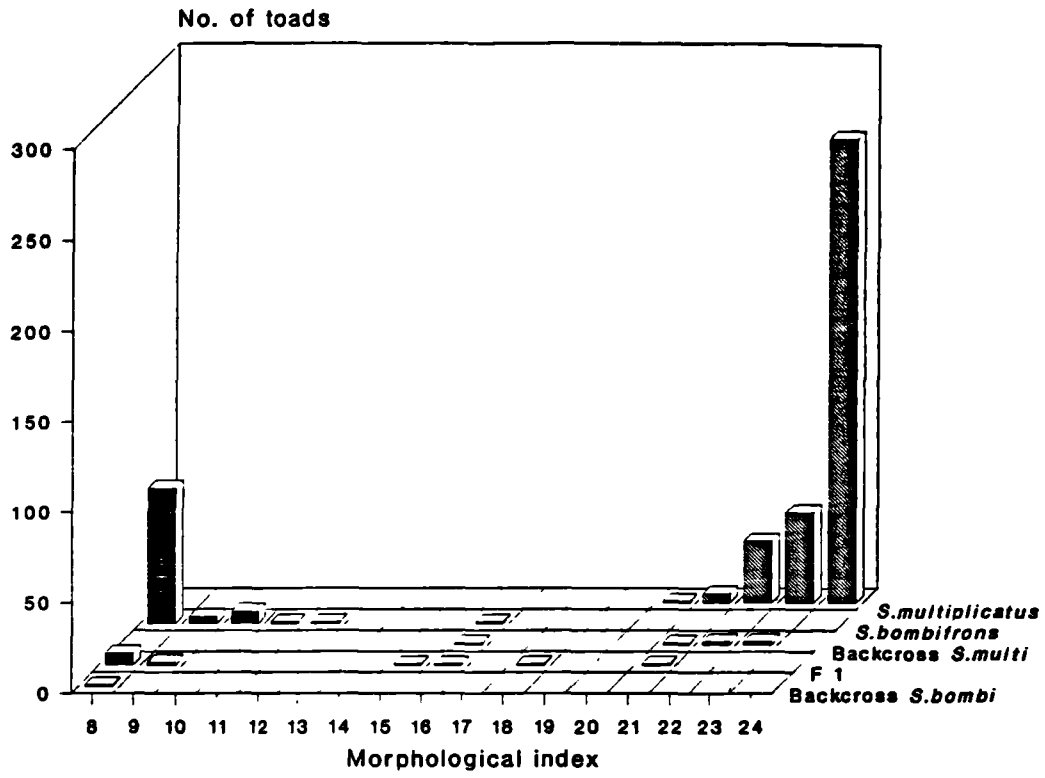

To Animas
Hwy 9
Arizona
New Mexico
Hwy 80
Portal
Rodeo
To Douglas
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
N
2km

Fig.2.2

A


No. of toads
300
250
200
150
100
50
0
S.multiplicatus
S.bombifrons
Backcross S.multi
F 1
Backcross S.bombi
8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Morphological index

B

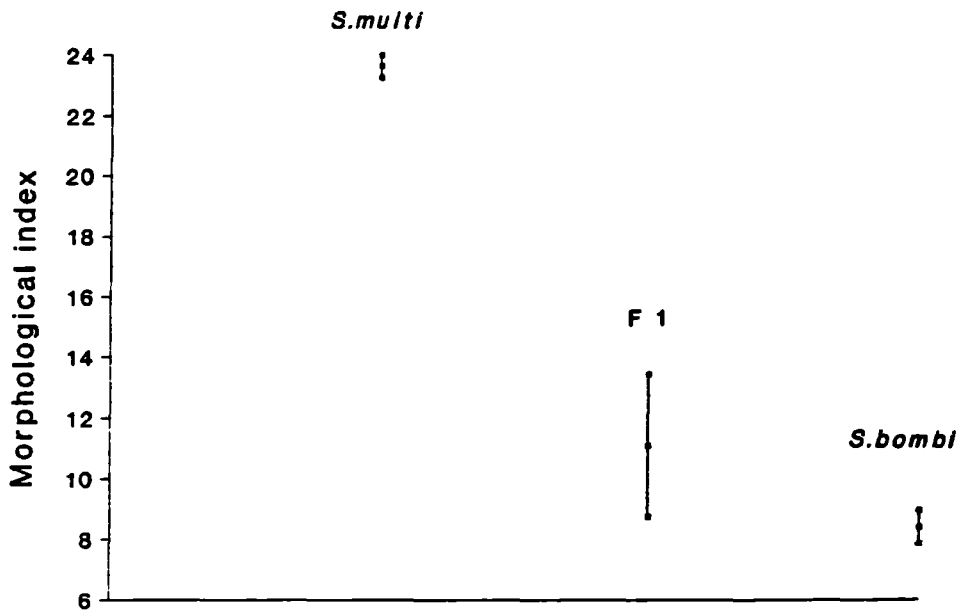
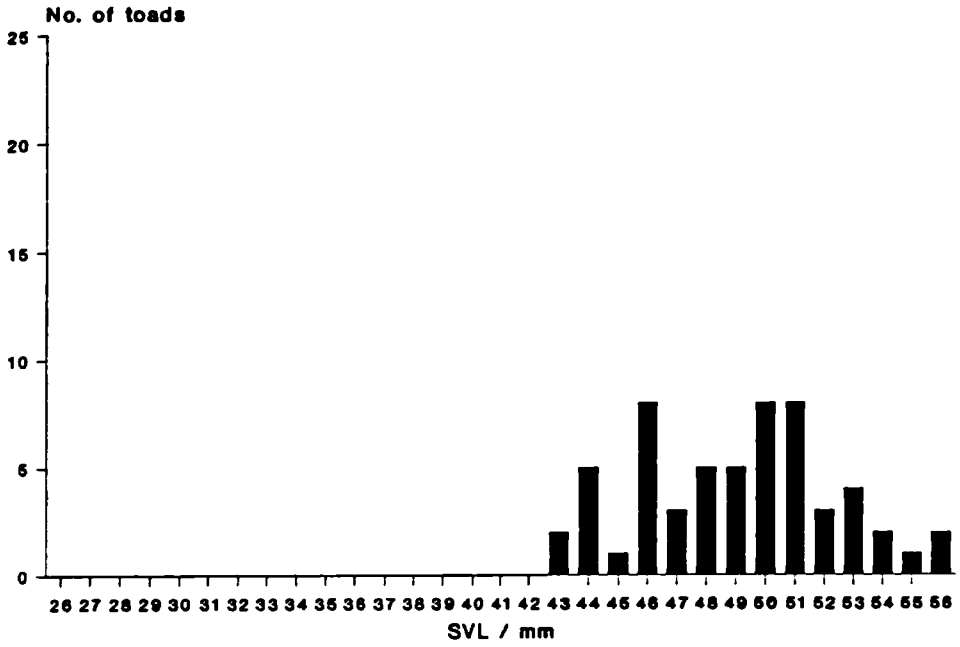

S.multi
F 1
S.bombi
Morphological index
24
22
20
18
16
14
12
10
8
6

Fig.2.3

A


No. of toads
25
20
15
10
5
0
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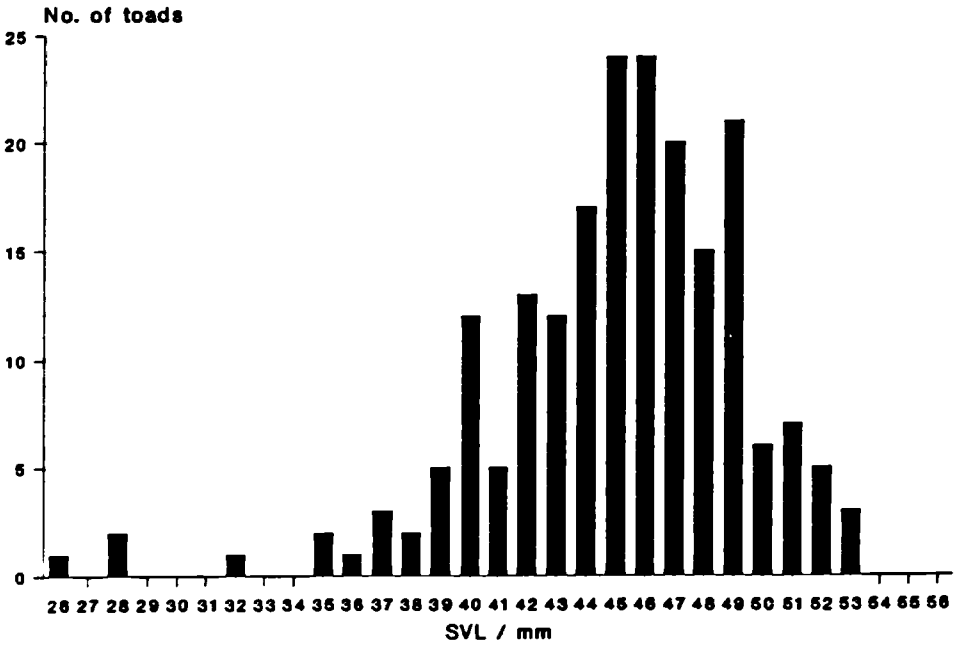
SVL / mm

B


No. of toads
25
20
15
10
5
0
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SVL / mm

Fig.2.4

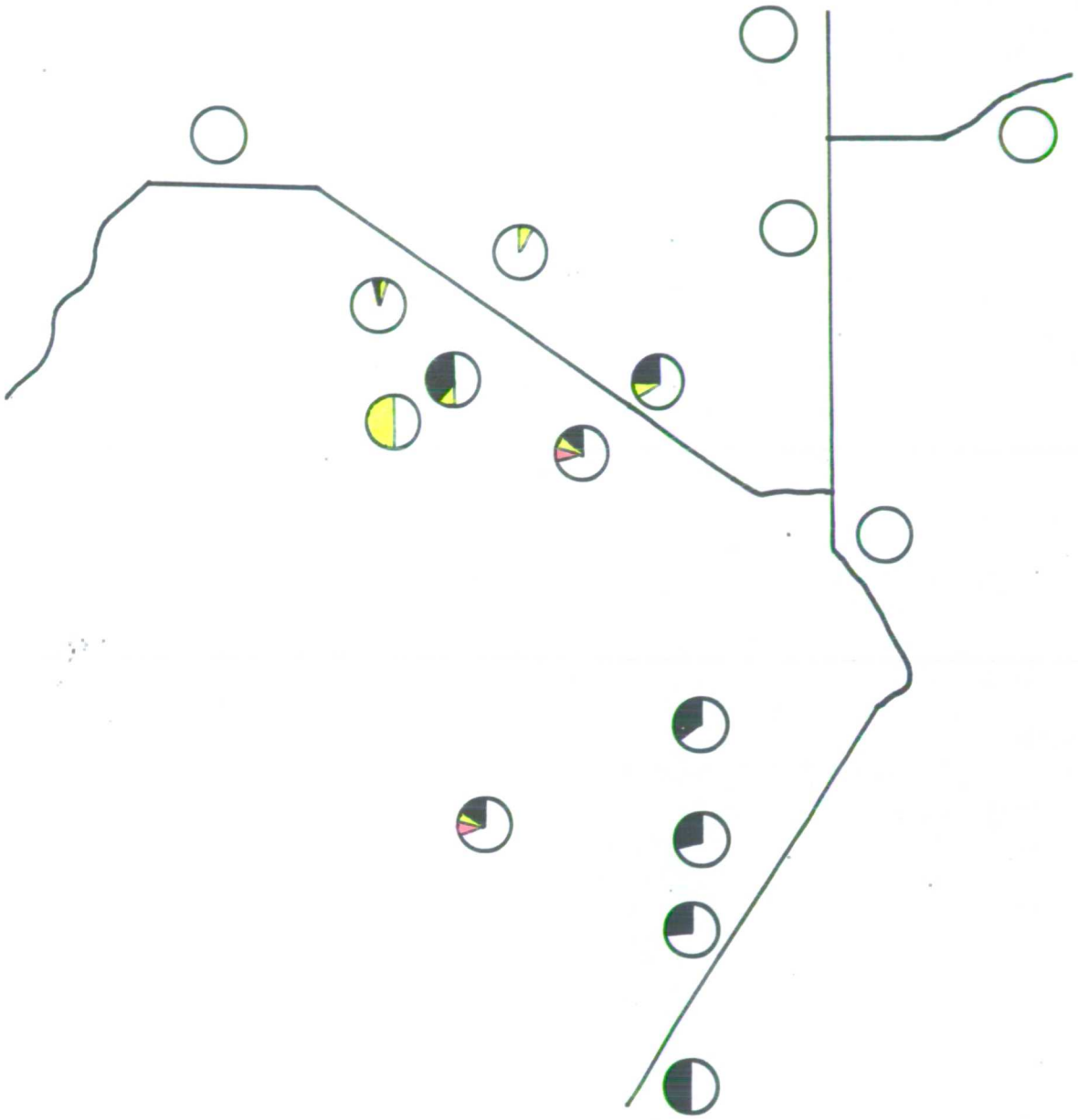

Fig.2.5

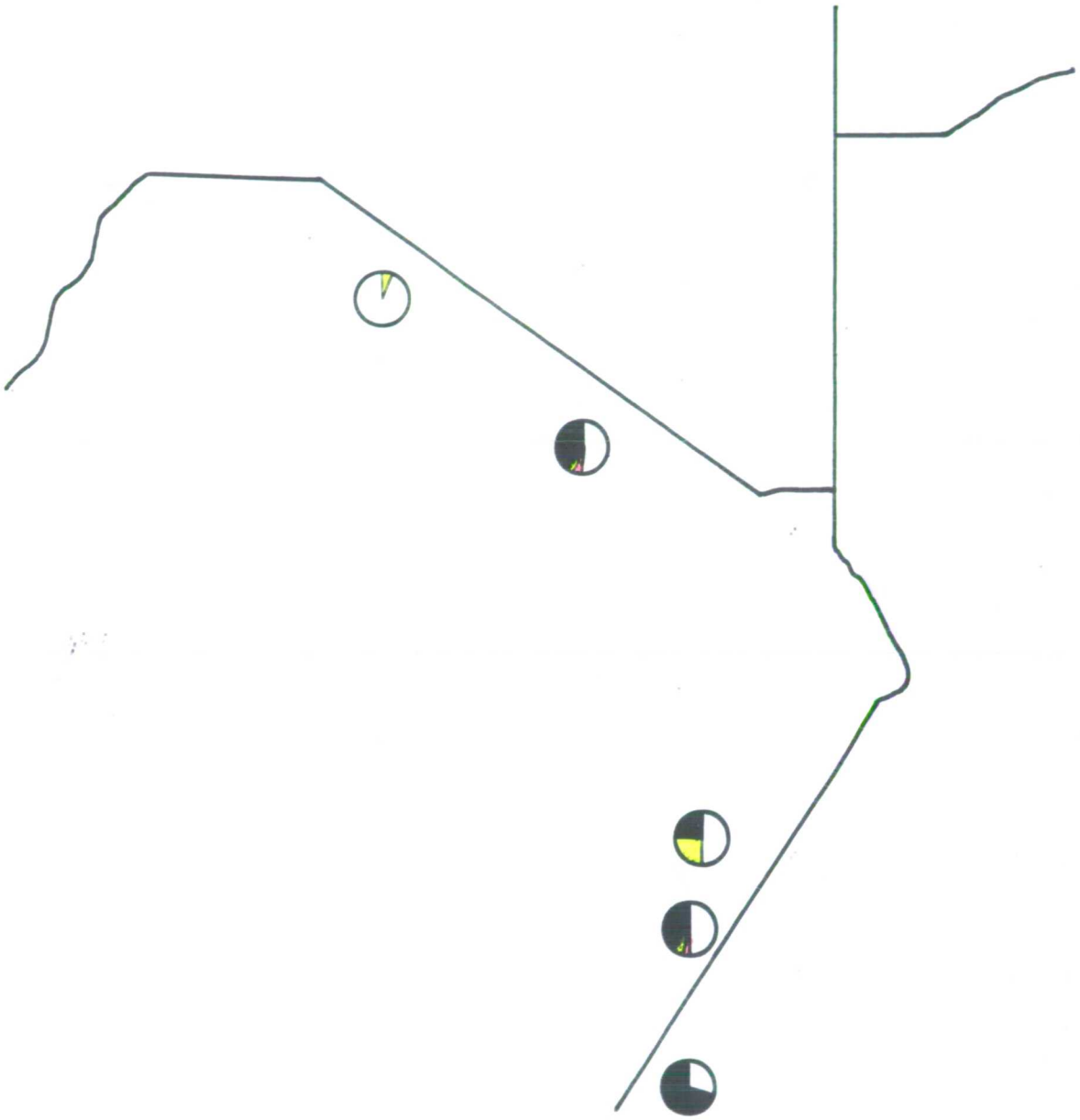

Fig.2.6

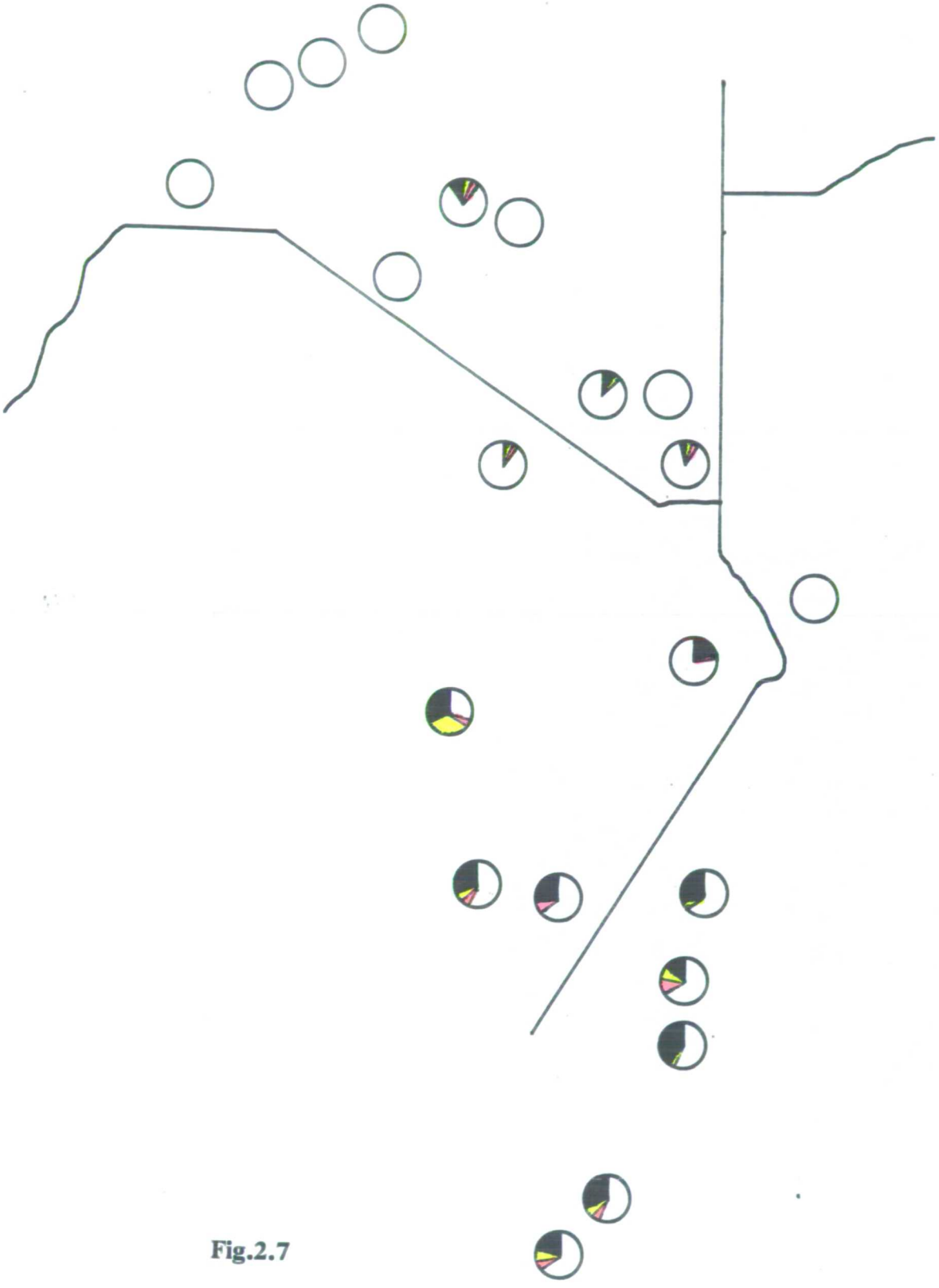

Fig.2.7

Chapter 3.

Neodiplorchis scaphiopodis (Rodgers, 1941) Yamaguti, 1963 (Monogenea: Diplorchiinae): Redescription, pre-migratory development, specificity and distribution in a host hybrid zone.

3.1 Abstract.

The polystomatid monogenean *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 has previously been recorded from two host species, the spadefoot toads *Scaphiopus bombifrons* and *S.multiplicatus*. Both hosts are sympatric in S.E. Arizona where there is a breakdown in reproductive isolation, creating a dynamic hybrid zone. Infection levels were documented in two consecutive years from a total of 154 electrophoretically-typed toads. Comparisons between host genotype/gender were based upon infections of juvenile and adult parasites in the respiratory tract and urinary bladder respectively. Frequency distributions of infection were characteristically aggregated for all genotypes, and respiratory infection levels reflected gender-related differences in host behaviour. Post-oncomiracidial development was documented to 24 days post-infection (p.i.) at variable temperatures. During this initial developmental period the reproductive system began differentiation. Moreover, the definitive haptor had fully differentiated prior to migration to the definitive site. A redescription of the adult is based on the largest sample of *N.scaphiopodis* yet described. Comparisons were based upon worms recovered solely from pure-bred hosts as no hybrids were infected with mature *N.scaphiopodis*. Morphological analyses (with particular reference to sclerites) revealed no specific differences between worms from either host genotype. This was confirmed by reciprocal cross-infection experiments, in which oncomiracidia successfully invaded, developed in the respiratory tract and migrated to the definitive site. A number of the worms recovered from the urinary bladder of heterospecific hosts reached sexual maturity, producing fully developed larvae. These data have established that the genus *Neodiplorchis* is represented by

a single species which infects both *S.bombifrons* and *S.multiplicatus*, contrary to the strict specificity proposed for anuran polystomatids. Preliminary cross-infections of *Pseudodiplorchis americanus*, the polystomatid monogenean infecting the sympatric spadefoot toad, *S.couchii*, were unsuccessful.

3.2 Introduction.

Ozaki (1931) described the first representative of a new monogenean genus following the discovery of *Diplorchis ranae* from the urinary bladder of Oriental anurans. This monogenean was distinguished by the possession of two condensed and symmetrically disposed testes, in addition to a elongated uterus accommodating numerous progeny. Subsequently, Lee (1936) described *Diplorchis nigromaculatus* from a ranid host in China and two similar flukes were recorded from spadefoot toads in Oklahoma, U.S.A. *Diplorchis americana* (Rodgers & Kuntz, 1940) was recovered from Couchs' spadefoot toad, *Scaphiopus couchii* and *Diplorchis scaphiopi* (Rodgers, 1941) from the Plains spadefoot toad, *Scaphiopus bombifrons*. Yamaguti (1963) reclassified these parasites into different genera within separate sub-families of the Polystomatidae. *D.ranae* and *D.americana* were distinguished from *D.scaphiopi* by the possession of follicular vitellaria. *D.ranae* was placed within the Diplorchiinae (haptoral hamuli present) and *D.americana* within the Beauchampiinae (devoid of hamuli). *D.americana* was assigned to a new genus, becoming *Pseudodiplorchis americanus* (Rodgers & Kuntz, 1940) Yamaguti, 1963 and on the basis of possessing vitellaria in two compact groups, *D.scaphiopi* Rodgers, 1941 was assigned to the Neodiplorchiinae, becoming *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963.

In their generic key of the Monogenea, Prudhoe & Bray (1982) classified all three species to the order Polyopisthocotylea, family Polystomatidae, sub-family Diplorchiinae Yamaguti, 1963. Differentiation of the Diplorchiinae from the Polystomatinae was based on the presence of two compact testes rather than the male gonad forming a reticular or follicular mass. Specific diagnosis was based on haptoral hamuli being absent in *Pseudodiplorchis*, and for *Neodiplorchis* and *Diplorchis*, where hamuli are present, differentiation was based on the vitellaria, being in two compact groups or extending along the intestinal caeca respectively.

Lamothe-Argumedo (1973a) re-described *N.scaphiopodis* from a new host, the Mexican mountain spadefoot toad, *S.multiplicatus* in Capulhuac State, Mexico. In a review of amphibian parasites in the State of Nebraska, U.S.A., Brooks (1976) recorded *N.scaphiopodis* from *S.bombifrons*. The studies of Tinsley and co-workers has formed the primary research into the biology of *N.scaphiopodis* and *P.americanus*. The restricted window for parasite transmission dictated by host ecology has been documented (Tinsley, 1982; Tinsley & Jackson, 1988) and the unique adaptations of *N.scaphiopodis* and *P.americanus* to overcome these pressures (Tinsley & Earle, 1983; Tinsley & Jackson, 1986).

The recovery of *N.scaphiopodis* from *S.bombifrons* and *S.multiplicatus* is of interest as strict specificity within anuran polystomatids has been proposed by a number of workers (Bourgat, 1977; Bourgat & Salami-Cadoux, 1976; Combes & Channing, 1978-1979; Euzet, Combes & Knoepffler, 1966; Kok & van Wyk, 1986; Murith, 1981). This assumption was based on morphological variation, in

conjunction with sparse experimental evidence (Combes, 1966, 1968) and, as yet, unpublished data referred to by Bourgat & Salami-Cadoux (1976) and Kok & DuPreez (1993). Moreover, a broader host specificity has been recorded by a number of authors for branchial worms (Kok & DuPreez, 1987; Maeder, 1973) and adults (Murith, 1981; Vaucher, 1990). The sympatric occurrence of two species in a single host species (Bourgat & Murith, 1980) and the syntopic occurrence of two genera within the same host (DuPreez & Kok, 1992) has also been documented.

Tinsley (1983) recorded that reciprocal cross-infections between heterospecific hosts (i.e. *N.scaphiopodis* to *S.couchii* and *P.americanus* to *S.bombifrons*/ *S.multiplicatus*) did not survive either in the field or laboratory. Simovich (1985) documented a dynamic hybrid zone between *S.bombifrons* and *S.multiplicatus* which changed both spatially and temporally. F_1 males are sterile but females backcrossed with either parental species (see Chapter 2). Host hybridisation will present *N.scaphiopodis* with an array of genotypes which may influence susceptibility to infection by host specific parasites. This provides an opportunity to examine the distribution of this monogenean in hosts of pure and hybrid genetic constitution. Field studies of wild populations of mice in Europe have documented overwhelming helminth burdens in individuals of hybrid genotype compared to pure bred hosts sampled from outside the zone (Sage *et al.*, 1986; Moulia *et al.*, 1991). A simular phenomenon has also been recorded for fish (Dupont & Crivelli, 1988). Alternatively, the work of Coustau *et al.* (1991) and LeBrun *et al.* (1992) indicates that a particular parental host genome may be able to resist infection by

specific parasites; therefore, the selective pressure may favour the non-susceptible genome (and their closely related hybrids), displacing the competing (susceptible) species.

By laboratory investigation, genetic predisposition of hybrid mice to specific helminths has been demonstrated (Moulia *et al.*, 1993; Munger *et al.*, 1989; Wassom *et al.*, 1973, 1974, 1986). Within a hybrid zone, predisposition to infection will affect the survival and competitive ability of a particular genotype, which will influence the genetic flow within the zone. Furthermore, it is conceivable that rather than a single species infecting both hosts, there may be two cryptic, species-specific helminths. A primary remit of this study was to test specificity by reciprocal cross-infections of electrophoretically-typed recipients with larvae taken from pre-typed donors.

There have been a number of studies concerning the post-oncomiracidial development of monogeneans, from both natural infections (Kearn, 1963; Llewellyn, 1959, 1960; Ogawa, 1984; Salami-Cadoux, 1975; Sproston, 1945; Thoney & Munroe, 1987; Wiskin, 1970) and laboratory investigations (Alvey, 1936; Frankland, 1955; Hoshina, 1968; Kasahara, 1967; Jackson & Tinsley, 1988; Tinsley & Owen, 1975). Sexual maturity typically involves the male reproductive system developing prior to the female system. However, Frankland (1955) found synchronous development of male and female systems in *Diclidophora denticulata*, and Tocque (1990) reported the precocious development of the female reproductive system in *P.americanus*. Moreover, the gyrodactylids are protogynous (Turnbull,

1956; Lester & Adams, 1974), with sexual reproduction occurring only after the birth of the first and second born daughters (Harris, 1993). The period from establishment to maturity varies widely between species: for *Dactylogyrus vastator*, the onset of egg production may occur after 4-5 days at 28-29°C (Paperna, 1963), while in extreme contrast *Polystoma integerrimum* develops to sexual maturity in synchrony with its host, at 2-3 years (Gallien, 1935).

Following invasion of the definitive host, helminths often have to undergo complex migrations to reach the definitive site, with maturation occurring either before or after establishment in the definitive site. Chappell (1982) proposed that, during development, sequential stimuli provided the triggers for migration, although Smyth & Halton (1983) recognised that the physiological processes governing maturation were not well understood. Sommerville & Rodgers (1987) suggested that the trigger for final development depended upon the temperature, physio-chemical and nutritional status of the host. These complex patterns of establishment, migration and maturity are present in the Monogenea, even though their life-cycles are direct. For example, the anuran polystomatids *Metapolystoma cachani* and 14 of the species of *Polystoma* recorded from Africa possess neotenic forms (Van Niekerk, 1992). Neotenics, resident on the tadpole gills, rapidly develop to sexual maturity and produce eggs at 2-4 weeks p.i., dying at host metamorphosis (Combes, 1968; Gallien, 1935; Kok, 1990; Kok & DuPreez, 1989). Typically inhabiting the same host are the 'bladder-destined' forms which exhibit a much slower developmental rate, moving from the gills to the urinary bladder, via the epidermis, at host metamorphosis (Combes, 1967). Although not

fully established, it has been suggested that the biochemical status of the host determines the developmental path taken (Murith, 1982).

Polystomatids may directly enter via the cloaca and initial development may occur within the renal system, for example *Protopolystoma xenopodis* (see Thurston, 1964; Tinsley & Owen, 1975) and *Eupolystoma alluaudi* (see Salami-Cadoux, 1975). Tinsley (1978) stated that *E.anterorchis* does not have an obligatory phase in the kidneys prior to development in the urinary bladder. Tinsley & Owen (1975) documented the primary development of the haptor and reproductive system of *Protopolystoma xenopodis* during an extended period of 2-3 months at 22°C in the kidneys, however, final maturation is not completed until entry into the definitive site. Entry into the definitive site may not be necessary for final maturation as Salami-Cadoux (1975) reported that *E.alluaudi* may begin egg production whilst in the kidneys, although these are stored in the uterus, with no oviposition prior to entry into the bladder.

Tinsley & Earle (1983) reported the unique life-cycle of *N.scaphiopodis* and *P.americanus* infecting spadefoot toads in Arizona. This is adapted to the terrestrial ecology of the host, with opportunities for transmission restricted to the brief breeding season, the only aquatic phase of the toads life-cycle. The oncomiracidia entered the respiratory tract via the nostrils, migrating through the mouth and glottis, to develop within the lungs. The invasion of the lungs remains unprecedented in monogenean biology. A return migration to the buccal cavity may begin after 3 weeks p.i., the worms having developed the basic structure of

the definitive haptor (Tocque, 1990). The migration route was established by Tinsley & Jackson (1986). At 4-7 weeks p.i. worms were recovered moving through the alimentary tract to the bladder, with host activity providing the stimulus to migrate. Those worms within host individuals which remained inactive were found arrested in the respiratory tract for up to 1 year p.i. Reproductive maturation was shown to be initiated in pre-migratory worms, with primordial cells developing as early as 2 weeks p.i. (Tocque, 1990).

Previous descriptions of adult *N.scaphiopodis* (Lamothe-Argumedo, 1973a; Rodgers, 1941) provided extensive morphological details but with no experimental comparison of specificity between potential hosts. Lamothe-Argumedo (1973b) expanded the original description of the larva of *N.scaphiopodis* by Rodgers (1941). Furthermore, Tinsley (1983a) commented on the phylogenetic relationships within the Polystomatidae based on oncomiracidial characters, with particular reference to the close relationships within the Diplorchiinae. To augment the observations made by Tocque (1990), a second remit of this study was to examine the pre-migratory development of *N.scaphiopodis*.

3.3 Materials and Methods.

3.3.1 **Field Studies.**

Fieldwork was based at the Southwestern Research Station (S.W.R.S.) of the American Museum of Natural History, Portal, Arizona in 1991 and 1992. The study area was located in the nearby San Simon valley. The valley lies between 1280-1520m above sea level (Dimmitt & Ruibal, 1980) and receives an annual rainfall of approximately 220mm, which is concentrated into monsoonal downpours during July to September (Ruibal, Tevis & Roig, 1969).

Ephemeral ponds are formed by the heavy rain and run-off from the surrounding land. The spadefoot toads, *Scaphiopus spp.* were recovered from a number of ponds in 1991 and 1992 (see Chapter 2, Fig.2.1). Following collection, toads were placed in plastic tubs containing moist potting compost, which prevented further transmission by *N.scaphiopodis*. Upon return to S.W.R.S. the animals were transferred to large aquaria (70x30x50cm), filled with moist compost to a depth of 15cm. Groupings were based on site and then further sub-divided by genotype once individuals were electrophoretically typed. In both years, a sub-sample of toads was dissected to document natural invasion levels (n = 113 and n = 30 respectively). Those remaining at the end of the fieldwork period were air-freighted to Q.M.W.

3.3.2 **Laboratory Maintenance of the Host.**

Using the same criteria for grouping, up to 30 toads were maintained in each rectangular plastic tank (80x50x40 cm) containing approximately 15 cm of moist

compost (John Innes No.1). The tanks were kept in constant temperature rooms. These insulated rooms contained thermostatically-controlled Frigia-Bohn heating/refrigeration units. Animals were exposed to slightly different regimes in each year which are detailed in Table 3.11. 25°C was used to maximise parasite growth and development, 20°C for infection and 15°C to minimise depletion of host resources during hibernation.

3.3.3 Host Feeding.

The toads were fed at intervals to maintain condition and stimulate respiratory worm migration (Tinsley & Jackson, 1986). Commercially supplied crickets (Monkfield Nutrition, Cambridge) were placed in large plastic arenas with up to 25 toads. The arenas were covered and the animals were allowed to feed to satiation, typically 2 to 3 hours. Any debris was washed from the toads prior to their return to the soil. Feeding bouts consisted of a minimum of 8 separate exposures to food, each separated by 48h.

3.3.4 Host Hibernation.

After periods of feeding the toads were maintained either in bulk, as noted above, or kept individually. Those animals which were segregated were done so on the basis of a hybrid genotype or a positive 'light-test' (see below). Each animal was placed in a 30cm section of underground drainage pipe (Wickes Cat #432-001). The ends were sealed with perforated 115 mm diameter plastic lids. The tubes contained a depth of approximately 20 cm of moist soil. The perforations in the lids allowed good percolation of water and ventilation of air.

Tubes were placed in groups of 15 to 18 in plastic tubs which had a 5 cm basal layer of soil. The soil was kept moist by regular watering, more frequently at higher temperatures.

3.3.5 A Visual Test for Infection.

Infection with adult *N.scaphiopodis* was assessed by holding toads up to a fibre-optic light source in a darkened room. The silhouette of adult worms could often be seen, as the toads have a delicate, transparent skin and large bladder volume. This method (referred to as 'light-testing') provided a rough measure of prevalence with infected toads removed from the main sample for dissection. Individuals which tested positive for the light-test were put into a 25^{0}C temperature regime to maximise worm growth and larval accumulation. These worms provided material for cross-infection experiments.

3.3.6 Laboratory Maintenance of Parasite Stages.

3.3.6.1 Collection of Oncomiracidia.

At dissection, adult worms were removed from the bladder and placed in a watchglass filled with 0.6% saline. The gut of each worm was ruptured with a needle and the haematin allowed to escape. The worm was then washed and placed in dechlorinated water. Further dissection released the encapsulated larvae which rapidly hatched. The intact haptor was fixed in a 10% formal saline solution for analysis of the hamuli. The oncomiracidia were gently taken up into a pasteur pipette and standard doses placed with pre-typed recipient animals (see section 3.3.6.2 below). For each worm the total numbers of oncomiracidia, developing

embryos and moribund larvae were recorded.

3.3.6.2 **Cross-Infections.**

Recipient toads were typed and light-tested prior to use. These toads were then exposed, under standard conditions, to oncomiracida of *N.scaphiopodis* from pre-typed hosts. Each recipient toad was placed in a 500ml beaker, containing 150ml of dechlorinated water and oncomiracidia pipetted below the water surface. The swimming action of the toads kept their skin moist, providing optimal conditions for oncomiracidial locomotion. The number of individual infective stages contained within each standard dose was dependant upon the availability of oncomiracidia. Oncomiracidia were pipetted directly onto the nose (for *P.americanus* and *N.scaphiopodis* preference experiments) or into the water (for single host *N.scaphiopodis* exposures). The history of all recipients was recorded from their point of capture, to assess any possible natural exposure to infection.

After 4 h (1-2 h for *P.americanus* exposures) at 20°C (used to simulate field temperatures), the toads were placed in individual, labelled tubes. The toads were maintained at 25°C (following Tocque & Tinsley, 1991b, 1994b). The number of oncomiracidia remaining in the infection vessel was recorded to estimate the success of invasion. Recipient toads were fed crickets in individual arenas 3 to 4 weeks p.i. to encourage respiratory worm migration (Tinsley & Jackson, 1986).

3.3.6.3 Transfer of Respiratory Worms.

Worms recovered from the respiratory tract, at dissection, were maintained in a 0.3% saline solution. Recipient toads were infected directly, by pipetting the worms into the mouth. Toads were placed in individual, labelled tubes and maintained at 25°C.

3.3.6.4 The Direct Transfer of Adult Worms to the Bladder.

Worms recovered from the bladder were maintained in a 0.6% saline solution. Recipient toads had their bladders emptied with 15cm of soft vinyl tubing gently inserted via the cloaca (external diameter 2.1mm, Portex Ltd.). A 2ml syringe was attached to one end of the tubing and a 5cm length of 1.65mm diameter cannula (Portex Ltd.) to the other. The worm was taken up, with a minimum volume of saline, into the cannula which was reinserted into the recipient toad's bladder. The recipient toad was kept in a crystallising dish for 1 h to check for expulsion of the worm. After this time each toad was placed in a marked tube and maintained at 25°C.

3.3.7 Host Necropsy.

Toads were anaesthetised in a 1% MS 222 solution (Sigma and Sandox Chemical Companies) for 15 minutes. A depth of 5mm was used to prevent entry of the anaesthetic into the respiratory tract. The snout-vent length (SVL) was recorded with vernier callipers to the nearest 0.5mm and body weight to 0.01g. A morphological index (described in Chapter 2) was taken.

The body cavity was opened by a mid-ventral incision into the abdomen, cutting through the skin and muscle layers. The heart was accessed by removal of the pericardium. The heart was lifted away from the viscera (to prevent contamination with tissue fluid) and an incision made in the aorta. A heparinised microhaematocrit tube (Bilbate Ltd., Daventry) was filled with blood, sealed with crystaseal and spun in a centrifuge at 1500g for 2 minutes. The packed cell volume (PCV) was recorded for each animal, measured as a percentage of the total fluid. The gonads, fat body, stomach, intestine and liver were weighed to the nearest 0.001g. In addition, the length of the intestine was recorded to the nearest mm. The urinary bladder, left and right lungs, alimentary tract, head and lower jaw were placed into separate petri dishes filled with 0.6% saline. Each was examined for *N.scaphiopodis*, with the nares, eustachian tubes, glottis, male vocal sac, stomach, intestine and rectum opened out to examine for juvenile stages as well as the definitive site, the urinary bladder. The numbers of worms at each site were recorded. All were fixed in a 10% formal saline solution, flattened under 22x22mm coverslips with pressure sufficient to display the hamulus profile.

3.3.8 Histology.

The majority of *N.scaphiopodis* recovered were fixed *in toto* for morphological (developmental) analysis. From storage in 10% formal saline, worms were washed in distilled water and transferred to alum carmine for a minimum of 18 hours. Specimens were again rinsed in distilled water and serially dehydrated in two changes of 70%, 90%, 95% and absolute alcohol. The worms were cleared in two changes of xylene, prior to mounting in D.P.X. or Canada Balsam. Morphology

was recorded with a Nikon Optiphot UFX-IIA microscope fitted with a camera lucida. Hamulus morphology was recorded by camera lucida drawings of hamuli dissected from the haptor and individually mounted. The length of the hamulus blade was measured with a curvimeter (constructed from graph paper - Chartwell) from drawings made at x100 magnification. The morphology of the marginal hooklets was restricted to hooklet I (nomenclature following Llewellyn, 1963) at x100 magnification.

3.4 Results.

3.4.1 **Infection levels.**

Juvenile and adult *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 were recovered from the spadefoot toads examined in this survey. Three distinct categories were defined for *N.scaphiopodis* infection. Group 1 refers to recently invaded stages in the respiratory tract, these worms having been acquired in the short period of spawning prior to collection. Group 2 refers to recently migrated worms in the urinary bladder, which may be successful invasions from spawning earlier in the year of capture or worms from the previous year, which arrested in the respiratory tract over winter and were stimulated to migrate in the year of sampling. The final category, group 3 refers to adult stages which have been harboured by the host for at least one year in the urinary bladder.

Infection levels of *N.scaphiopodis* recovered from *S.bombifrons*, *S.multiplicatus* and their hybrids are summarised in Table 3.1. For *S.bombifrons*, recent invasions in the respiratory tract (group 1) ranged in prevalence from 23.1 to 50.0% (1-16

worms/host) over the two years of sampling, group 2 varied between 8.3 and 15.4% (1-7 worms/host) and group 3 ranged from 7.7 to 16.7% (in single burdens only). For *S.multiplicatus*, the prevalence of group 1 worms ranged from 58.8 to 60.0% (1-32 worms/host), group 2 varied between 2.9 and 11.8% (1-2 worms/host) and group 3 ranged from 0 to 19.0% (1-4 worms/host). No adult infection was recovered from hybrid genotypes, with only group 1 worms recovered in 1991, at a prevalence of 50.0% and a maximum burden of 11 worms/host.

In 1991, similar infection levels of group 1 worms were recorded for all host genotypes. However, for group 2 there was wider variation, particularly in prevalence. Adult parasites (group 3) had comparable prevalences between host species, but infected *S.multiplicatus* typically harboured heavier burdens. In the smaller host samples examined in 1992, there was generally more variation between *S.bombifrons* and *S.multiplicatus* for all three groups than in the previous year and the single hybrid toad was uninfected. Frequency distributions indicate that, for the largest host sample (*S.multiplicatus*), both respiratory and bladder infections correspond to an overdispersed distribution (Fig.3.1). From the smaller samples of *S.bombifrons* and hybrid hosts, respiratory infections were correspondingly limited, fitting no clear pattern (Fig.3.1A), this was also the case for *S.bombifrons* bladder infections (Fig.3.1B). Comparisons on the basis of host sex were made using the largest pure species sample, *S.multiplicatus* collected in 1991. Of the 105 individuals dissected 30.5% were females. Males harboured both higher prevalences and intensities of juvenile and sub-adult worms, however, they

also possessed lower infection levels of gravid adults (Table 3.2).

3.4.2 Developmental rate of pre-migratory *N.scaphiopodis* in the respiratory tract.

Dissection of 154 spadefoot toads collected from breeding ponds in 1991 and 1992 provided a record of the development of juvenile *N.scaphiopodis* in the respiratory tract, prior to migration to the definitive site. The toads were maintained at variable temperature, a mean of 22.2^{0}C (range 17-29^{0}C) in 1991 and a mean of 21.9^{0}C (range 18-28^{0}C) in 1992.

Following invasion via the nostrils (as described by Tinsley & Earle, 1983), the worms rapidly moved into the oral cavity (Fig.3.2A & B). The nares and sinuses harboured only 7% of the worms recovered in the first day p.i., the remaining 93% having already entered the mouth. In the initial 4 days p.i., there was a clear pattern of migration from the point of entry, through the mouth and glottis, into the lungs (Fig.3.2A-C). The lungs were invaded as early as 2 days p.i., with a single worm having successfully reached this site. Although all sites in the respiratory system harboured worms, between 4 to 15 days p.i. the majority were recovered from the lungs. There was no clear pattern to the return migration to the mouth, prior to migration to the definitive site (as described by Tinsley & Earle, 1983).

Morphological development.

A suite of measurements was recorded to document the development of

N.scaphiopodis. Total body length, haptoral length and pharynx length were the most consistent measure of developmental rate with the lowest coefficient of variation. Whilst in the respiratory tract the worms increased in overall dimensions and there were a number of morphological changes/developments.

Total body length and Pharynx length.

During the period of migration to the lungs (1-6 days p.i.), all the worms had taken a blood meal (described below), however, there was no discernible growth prior to 9 days p.i. (Fig.3.3). From 9 to 19 days p.i., there was an overall increase in the size of worms. There was some degree of variation during the experimental period, particularly at 6, 11, 16 and 24 days p.i. Pharyngeal length exhibited a similar pattern to total body length, with no perceptible growth to 7 days p.i. after which there was a variable, but clear increase until 19 days p.i. (Fig.3.4). For both total body length and pharyngeal length, the fluctuations in the plots are discussed below in relation to sample size and histology.

Haptoral dimensions.

The oncomiracidum bears 16 marginal hooklets but no suckers on the muscular, clasping haptor. It is these hooklets that provide the initial attachment for the parasite (their dimensions are described in section 3.4.4 below). Haptor length follows a similar pattern to total body length until 11 days p.i. (Fig.3.3). Between 10 and 15 days p.i., there was no apparent increase in size, after which there was a overall increase to 24 days p.i. As above, the fluctuations in the plots are discussed below in relation to sample size and histology.

After the beginning of blood feeding (2 to 5 days p.i.) deep staining cell masses circled hooklets III, IV and V (nomenclature following Llewellyn, 1963). At 1 week p.i., small projections were apparent above the ventral surface of the haptor. The suckers first became apparent after 9 days, with equivalent development of all 3 sucker pairs (posterior, middle and anterior). During the subsequent pre-migratory developmental period (9-24 days p.i.), the hooklets became encircled by increasingly large, muscular suckers. By the time worms were migrating back to the mouth, these functioned independently and the haptor itself had progressed from a cup-shaped disc to a flat, sub-hexagonal disc (Fig.3.6). Initially, the hamulus primordia were twice the size of the marginal hooklets, at approximately 50μm in length (Fig.3.5A, 2 days p.i.). The hamuli grew steadily (Fig.3.5A-H) and by 24 days p.i., were clearly defined and had, on average, doubled in size from the original primordia.

Intestinal arrangement.

The morphology of the gut was characterised by two broad, simple caeca, bifurcating immediately posterior to the pharynx and reuniting in the haptor. All the worms recovered 1 day p.i. had opaque guts and had not begun feeding on blood. The commencement of blood feeding at 36h p.i. was indicated by a slightly pink pigmentation of the caeca. This became bright red between 3 and 6 days p.i. and after 7 days p.i., all worms possessed deep red to brown gut contents, eventually becoming almost black by their return migration. Although all the worms recovered from the lungs had fed on blood, those worms remaining in the

oral cavity and nasal passages between 2 and 5 days p.i. typically had not blood-fed, with only 1/66 (1.5%) having ingested a blood meal. By 24 days p.i. a number of shallow diverticula had formed but no anastomoses were recorded (Fig.3.6).

Reproductive system.

The reproductive system was characterised by a mass of primordial germ cells which formed a deeply staining region immediately posterior to the caecal bifurcation at 10 days p.i. A fully developed pre-migratory worm (Fig.3.6) possesses two clearly defined lateral bodies (corresponding to the testes) and a central body (occupying the same area as the ovary in adults), associated with the medial, descending branch of the uterus.

3.4.3 Post-migrational development.

A limited number of worms was recovered from the bladder, with the sexually mature individuals described below. Those which had recently migrated were only slightly larger than pre-migratory forms. The major difference was the further development of the reproductive system, post-migratory forms possessing distinct testes, ovary and the descending limb of the uterus. However, insufficient oncomiracidia and the lack of successful control animals from Section 3.4.5 (described below) did not allow for sufficient laboratory-controlled infections, which could provide a full description of *N.scaphiopodis* maturation in the definitive site.

3.4.4 Description of *N.scaphiopodis*.

Observations are based on a sample of 73 whole mount specimens of sexually mature *N.scaphiopodis* recovered from the urinary bladder of *S.multiplicatus* at burdens of 1-7 parasites/host. The measurement of the larval sclerites was based on larvae and adults, recovered from typed hosts. All measurements are in mm (except where indicated) with the range, mean and standard deviation recorded. A summary of the main characters are recorded in Table 3.3, which also incorporates previous data published by Rodgers (1941), Lamothe-Argumedo (1973a) and Brooks (1976).

Overall body form lanceolate with terminal opisthaptor (Fig.3.7A). The parasite measures 2.21-7.35 (4.88 +_ 1.28) in total body length with a maximum width of 0.80-2.08 (1.31 +_ 0.31). Width at the level of the vaginae measures 0.54-1.14 (0.78 +_ 0.12). The haptor ranges from 0.46 to 1.44 (0.95 +_ 0.21) in length and 0.76 to 1.88 (1.36 +_ 0.27) in width. The overall haptor length to total body length ratio is 0.19. Two pairs of eyespots persisting in the adult.

Haptor.

This sub-hexagonal disc bears three pairs of muscular suckers, equidistant around the periphery. A single pair of hamuli is situated between the posterior sucker pair. Marginal hooklet I ranges from 26 to 28μm (27 +_ 1μm) in length, with a total length to handle length ratio of 2.26. Marginal hooklets III, IV and VI are incorporated into the posterior, middle and anterior suckers respectively (nomenclature following Llewellyn, 1963). Hamulus length ranges from 236 to

482um (386 +_ 60um). Pooled data for the three pairs of haptoral suckers produces an overall diameter of 0.14-0.32 (0.24 +_ 0.04).

Intestinal arrangement.

A muscular oral sucker is found at the anterior extremity, the mouth opening in the centre. The pyriform muscular pharynx lies just behind the mouth. Pharynx ranges from 0.09 to 0.30 (0.15 +_ 0.03) in length and from 0.07 to 0.21 (0.11 +_ 0.02) in width. The intestine bifurcates just behind the pharynx (no oesophagus is present) and possesses numerous branched and unbranched lateral diverticula which may extend deep into the haptor, where the caeca reunite. Between 7 and 13 diverticula may anastomose, typically in the central body, with sectioned material revealing branches in close association with the folds of the uterus (Fig.3.7C).

Reproductive system.

The overall structure of the reproductive system is displayed in Fig.3.7B and is as described by Rodgers (1941) and Lamothe-Argumedo (1973a). Two elongate testes are arranged symmetrically and laterally, situated in the anterior fifth of the body. The testes measure 0.34-1.5 (0.71 +_ 0.25) in length and 0.13-0.47 (0.29 +_ 0.07) in width. Vas efferentia project into the centre of the body, meeting to form the vas deferens which passes anterior to the genital pore. The genital pore lies in a median position, slightly anterior to the level of the vaginae and bears 8 spines with a mean length of 13-18μm (15 +_ 1μm).

The ovary is pre-testicular, ovoid and sub-medial, either on the right or left, measuring 0.13-0.28 (0.20 +_ 0.04) in length and 0.08-0.18 (0.14 +_ 0.02) in width. The oviduct is short, entering the ootype, which is surrounded by a prominent Mehlis' gland, slightly anterior and dorsal to the junction of the vas efferentia. Vaginae paired, with distinct lateral apertures, lying immediately posterior to the genital pore. A genito-intestinal canal joins the oviduct and intestinal caecum, on the ovarian side. Vitelline glands are represented by two ovoid, compact bodies lying symmetrically and lateral to the ovary. The vitellaria measure 0.13-0.24 (0.17 +_ 0.03) in length and 0.07-0.19 (0.11 +_ 0.03) in width. Vagino-vitelline ducts, unite in the mid-body prior to joining the oviduct above the ootype. The intricate uterus forms a large loop, with the descending limb forming a slender tube running medially from the ootype to the haptor, its path occasionally meanders, broadening to accommodate embryos. The ascending limb possesses extensive transverse loops which pack the majority of the inter-caecal region (Fig.3.7B). Those progeny in the distal section of the uterus are fully-developed oncomiracidia ensheathed within a thin, membranous sac. In this study, a mean of 13.5 (S.D. = 22.2) fully-developed oncomiracidia and 105.8 (S.D. = 57.3) developing embryos were recorded.

In addition to the cross-infection experiments described below, a number of morphological comparisons were made between worms recovered from *S.multiplicatus* and *S.bombifrons*. Table 3.4 summarises the mean, standard deviation and range of body dimensions recorded for worms recovered from each pure host genotype. Overall, those worms recovered from *S.bombifrons* were

typically larger and the greatest variation fell exclusively within the largest sample (*S.multiplicatus*). Gross hamulus morphology is consistent between worms recovered from both hosts at the same locality (Fig.3.8A & B), variation between individuals within one host species being greater than between host genotypes. Geographical heterogeneity is evident between worms taken from both *S.bombifrons* (Fig.3.8B, C, D & F) and *S.multiplicatus* (Fig.3.8A & E). The relationship between total hamulus length and mean blade length (following Vaucher, 1990) indicates no clear separation between worms recovered from both host species, however, the largest hamuli were from *S.bombifrons* derived worms (Fig.3.9). The hamuli used in this analysis were taken from those worms dissected for oncomiracidia and within this sample, the *S.bombifrons* derived worms were the oldest worms. Following Murith (1981a), the total length of larval hooklet I (b) was plotted against the length from the handle to bifurcation point of the guard (a) but there was no separation between the samples by species (Fig.3.10). Comparisons of marginal hooklets between hosts recovered from Arizona (this study), Texas and Kansas (R.C. Tinsley, personal collection) and that portrayed by Rodgers (1941) indicated no variation in gross morphology or size.

3.4.5 Oncomiracidial cross-infection of *N.scaphiopodis* to heterospecific hosts.

A summary of the 100 single host cross-infections and controls is presented in Table 3.5A. Experimental work was concentrated at Q.M.W. with 89/100 exposures undertaken in London. In all, 68 heterospecific hosts and 32 conspecific controls were exposed to a total of 8458 infective stages of *N.scaphiopodis* from pre-typed donors. By counting the number of oncomiracidia left in the water after

the experimental period, it was estimated that 77.0% (S.D.= 18.1%) and 79.2% (S.D.= 16.8%) of oncomiracidia successfully entered recipient toads in heterospecific crosses and controls respectively. At dissection, only 42 active worms were recovered from the urinary bladder 7-26 weeks p.i. at 25°C (a single worm which had recently died was recovered from the control in infection #100). This represents 0.6% of all invasions estimated to have successfully entered the 84 recipients (78.4%, S.D. = 18.1) dissected 7-26 weeks p.i.

In 12/100 infections, the host died prior to the completion of the experimental period (7 cross-infection & 5 control animals), and in 9/100 infections the hosts already harboured gravid *N.scaphiopodis*. These worms may have been a source of autoinfection and the exposures were therefore considered void. In 36/68 (52.9%) of heterospecific crosses and 15/32 (46.9%) of controls, no worms were recovered at dissection. Apart from the 31 pure-bred *S.bombifrons* and 64 pure-bred *S.multiplicatus*, 3 F_1 hybrids and 2 backcross-*S.multiplicatus* were exposed to oncomiracidia. However, experimental infections were only recovered from pure-bred hosts. Toads which harboured worms considered to be the result of experimental infection accounted for 33/100 individuals. These successful cross-infections and a number of controls are described below.

Initial cross-infections (#1-16, Table 3.5A) indicated that *N.scaphiopodis* could invade heterospecific hosts and develop to a pre-migratory form. Recipients were either collected in 1990 and kept at Q.M.W. for 10 months (see Fig.3.11) or collected from the road early in the field season and maintained in the laboratory

for a short period prior to exposure. Therefore, natural infections were easily distinguished due to their advanced state of development in comparison to experimental infections. Primary exposures (#1-9, Table 3.5A) indicated that, in heterospecific crosses, larvae had an equivalent distribution in each area of the respiratory system as controls. Moreover, in both cross-infections and controls, blood feeding began at approximately 36 hours p.i. Hosts dissected at 3 weeks p.i. (#10-16) harboured worms which had dark brown gut contents and a cup-shaped haptor possessing six small, incompletely developed suckers. As worm burdens varied widely, with no clear pattern, it was at this point that the oncomiracidia remaining in the water after the experimental period were counted, to estimate invasion success. Of the 8 multiple host exposures summarised in Table 3.5B, only the short term exposures retained their infection. Exposure #1 suggests that when allowed a choice of conspecific and heterospecific host no preference for conspecificity was exhibited by *N.scaphiopodis*. In addition, at 1 week p.i. all worms had fed on blood and by 2 weeks p.i., the worms had developed small protuberances at the site of each of the six haptoral suckers, confirming the initial development provided by the single host exposures. From these data (initial single exposures #1-16 and multiple exposures #1-3) it was assumed that, for cross-infections, migration to the definitive site was possible. Therefore, subsequent experimental protocol was based on documenting whether migration occurred and if so, would the worms reach sexual maturity in the urinary bladder of a heterospecific host.

At 7 weeks p.i., cross-infection #20 (undertaken from October to November 1991) harboured a single worm in the bladder, which possessed deeply staining regions in the central and lateral body. The central area corresponded to the ovary and Mehlis' complex and the lateral areas to the testes, vaginae and vitellaria. The descending limb of the uterus was also beginning to form. The recipient host was collected in 1990, provided with 3 extended bouts of feeding and was light-tested on 3 occasions. Therefore, any previous infection will have had repeated stimulus to migrate (if arrested) and a maximum of 9 months at 20-25°C to develop prior to experimental use (Fig.3.11). Unfortunately, no worms were recovered from the controls; however, the recovery of the worm from toad #20 suggested that sexual maturity was possible in heterospecific hosts.

At 9 weeks p.i. cross-infection #30 (undertaken from October to December 1991) harboured a single worm which was recovered from the mouth. This pre-migratory form stained deeply in the region directly posterior to the intestinal bifurcation. This is the position of the reproductive system in sexually mature adults, however, this area had not begun to differentiate and consisted of primordial germ cells. The recipient host was also collected in 1990 and experienced the same maintenance regime as host #20. The control (#31) was also infected with a single worm, of equivalent development, recovered from the mouth. This host was a juvenile collected in 1991, maintained at 20°C and fed over a 3 week period in September (Fig.3.11). It is proposed that both worms were arrested, due to inadequate stimulus to migrate.

To assess whether sexual maturity was attained in heterospecific hosts, the remaining 67 toads (#32-100) were dissected between 12 and 26 weeks p.i. At 12 weeks p.i., 2 worms were recovered from cross-infection #36. A single arrested worm was found under the tongue and was of equivalent proportions and development to the worms collected at 9 weeks p.i. A slightly larger, recent post-migrant was recovered from the bladder. In this worm, the primordial germ cells of the reproductive system had begun differentiation, with distinct testes, ovary and descending limb of the uterus. At 13.5wks p.i. (#40) a single recent migrant was recovered from the bladder. This worm had recently fed (indicative for *P.americanus* of arrival at the definitive site: Tocque, 1990), and was at a similar state of development to the bladder worm of #36. However, in both cases, no embryos had been formed. For both #36 & 40, the recipient hosts were collected in 1991 and used for experimental purposes from August to December 1993. Therefore, these toads had 2 feeding bouts and experienced temperatures of 20^{0}C for 5 months prior to use (Fig.3.11).

The production of embryos was first noted in the control (#43) at 14 weeks p.i., this host harboured 4 worms, of equivalent size, in the bladder. A mean of 43 embryos (S.D. = 15) were formed but there were no larvae. Surprisingly, the worm recovered from control #52 at 15 weeks p.i., had not produced any embryos. A single worm was recovered from the bladder of heterospecific host #47 at 15 weeks p.i. This worm contained 6 fully developed larvae and 153 embryos in the uterus. The hosts used for infections #43, 47 & 52 were all collected in 1991 and exposed to infection in September/October 1992. Preceding

exposure to infection, they had one feeding bout and were light-tested on 2 occasions, therefore, any natural infections will have had a maximum of 3 months at 20^{0}C to develop prior to experimental use (Fig.3.11).

Also at 15 wks p.i., cross-infection #56 had an arrested pre-migratory worm under the tongue but no worms in the bladder. Five recent migrants were recovered from the bladder of cross-infection #55, all possessing the initial differentiation of the reproductive system. The descending limb of the uterus was clearly visible in all 5, but they contained no embryos. Cross-infection #57 harboured a single worm in the bladder, which was larger than those from #55. The reproductive system was distinct and the uterus contained 34 embryos. This particular infection indicated that sexual maturity and the onset of embryo production may occur in *N.scaphiopodis* at approximately 4 months following exposure to oncomiracidia. In addition, control host (#65) was found to have 3 pre-migratory worms under the tongue and 13 worms in the bladder, the latter having produced a mean of 44 embryos (S.D. = 12). Infections #55, 56, 57 & 65 may provide the most reliable indicator of the development of *N.scaphiopodis* at 15 weeks p.i., as the recipient hosts, all 1991 collections, had undergone a longer maintenance regime in the laboratory (identical to #36/40 described above) in comparison to #47. Therefore, if a natural infection was present the differences in development would have been distinct.

Worms were recovered from two heterospecific hosts (#68, 70) at 16 weeks p.i. The former harboured 3 worms, all recovered from the bladder. They contained

16, 0 & 0 fully developed larvae and 160, 115 & 81 embryos respectively. Host #70 had a single infection, also in the bladder and contained 157 embryos in the uterus. Control #75 was found to have a single bladder worm, but this was smaller than those of equivalent age from the heterospecific crosses and contained just 7 embryos. The recipient toads were collected in 1991 and utilised for experimental purposes at the same time as infections #43, 47 & 52 (described above). A single control was infected at 17 weeks p.i. (#82) harbouring a single worm, containing 71 larvae and 105 embryos. The recipient was also from the 1991 collection but not exposed to infection until December 1992.

A number of animals were exposed to infection just before fieldwork in 1992. As dissection prior to departure would have not allowed the worms to reach sexual maturity, they were dissected upon return (20-26 weeks p.i.). Cross-infection #84 was dissected at 20 weeks p.i., with a single worm recovered from the bladder, bearing 150 fully developed larvae and 104 embryos in the uterus. Host #86 also had a single worm in its bladder containing 64 larvae and 134 embryos. No equivalent infection was recovered from the control hosts. These hosts (#84, 86) were collected in 1990, fed during 3 feeding bouts and light-tested on each occasion. Experimental infection was undertaken in April 1992, thus any natural infections will have had a maximum of 9 months to develop at 20-25°C prior to experimental use (Fig.3.11).

Hosts #96, 98 & 100 were all control animals utilised between April and September 1992. Toad #96 harboured 13 worms in the bladder and although no

fully developed larvae were present, a mean of 216 embryos (S.D. = 66) had been produced. The 2 worms recovered from #98 had also produced a large number of embryos (287 & 321). The single worm from toad #100 had recently died, and although intact, disintegrated during fixation. These controls (#96, 98, 100) all utilised hosts with differing histories. Recipient #96 was exposed to one feeding bout and was light-tested twice, thus any natural infection will have had a maximum of 3 months at 20°C to develop prior to experimental use (Fig.3.11). Host #98 experienced the same laboratory regime as #96. However, as this was a juvenile collected in 1991, it should not have not entered a breeding pond and thus have had no exposure to infection. The final host (#100) was also a juvenile but collected in 1990.

By use of the light-test, only 9% of recipients were found to harbour pre-existing infections, although in nature this figure may reach nearly 20% (Table 3.1). Those exposures where pre-existing infections were identified (see Table 3.5A), all were clearly at a state of development too far advanced for 4 months p.i. and could therefore be discounted. The success rate for those cross-infections and controls from which experimental infections were recovered was 79.3% (S.D. = 16.9). This would suggest that oncomiracidial quality was not a factor in the successful establishment of worms. No correlation was found between host body weight, SVL or PCV and the number offspring produced by individual worms at the same age p.i.

3.4.6 Transfer of *N.scaphiopodis* respiratory stages.

In an attempt to increase the numbers of oncomiracidia available, all transfers of respiratory worms were to conspecific hosts. Recipient toads were light-tested but may have had recent exposure to oncomiracidia. In total, 12 transfers were made with 2 hosts harbouring gravid bladder worms at dissection, 4 were uninfected and 6 died prior to dissection 6-8 months p.i.

3.4.7 Transfer of adult *N.scaphiopodis* to the urinary bladder of conspecific and heterospecific hosts.

Conspecific transfers involved 11 toads, each receiving 1-3 non-gravid adults. Infection periods ranged from 5-6.5 months during which 7 hosts died, 3 were uninfected at dissection and 1 host harboured a large adult worm, which had recently died. Heterospecific transfers also involved 11 toads, all receiving 1 non-gravid adult each, with infection periods from 2-5 months, 7 hosts died, 3 were uninfected and 1 host harboured a single worm. This worm was active and had developed 20 embryos in the 9 weeks since transfer. The host had been returned from the field in 1990, fed twice and kept at 20^{0}C for 3 months and at 15^{0}C for 4 months prior to experimental use (Fig.3.11).

3.4.8 Cross-infections of *P.americanus* to *S.bombifrons* and *S.multiplicatus*.

Laboratory exposures of *S.bombifrons* and *S.multiplicatus* to the oncomiracidia of *P.americanus* are summarised in Table 3.6A. Recipient toads were maintained in hibernation for at least 6 months prior to experimental use. Initial exposures were observed with a dissection microscope and illuminated by a fibre-optic light

source. Oncomiracidia were pipetted directly on to the snout of the recipient toad and although no quantitative analysis was undertaken, a number of observations were made. A minority of oncomiracidia moved directly to the opening of the nostril and remained attached by their haptor whilst executing a probing action into the periphery of the nostril. Few oncomiracidia entered the nostrils after this action, most moving away. Others upon encountering the nostril, circumnavigated the whole or part of the aperture, again only to move away. The majority of the oncomiracidia were eventually found in the water, swimming vigorously and after making contact with the host did not reattach.

Of the 4 heterospecific exposures which were observed, all the worms entering the nostrils were recovered after 1d and 7d but none were found after 14d. Infections resulting from oncomiracidial invasion were recorded to a maximum longevity of 7d p.i. (Table 3.6A). Blood feeding began at 2d p.i. (3/12 worms) although not all worms recovered at 7d p.i. had ingested blood (1/4 worms). Gut contents remained bright red throughout the experimental period. A single control for the oncomiracidial infections was undertaken, with an individual *S.couchii* exposed to 160 oncomiracidia for 1h. The behaviour of invading oncomiracidia followed the pattern described by Tinsley & Earle (1983). After 1d p.i., 61 worms were recovered from the nares and mouth (no blood feeding had occurred).

Blood-fed worms recovered from the nares and mouth of a single host dissected at 2d p.i. were transferred to the buccal cavity of another host for 3 d (5d p.i.). A single worm was recovered and transferred to a further 4 hosts, the last successful

recovery was at 10d p.i. This worm represented the longest surviving worm which had been allowed to develop only in heterospecific hosts.

The transfer of respiratory worms from *S.couchii* to the buccal cavity of *S.bombifrons* and *S.multiplicatus* is summarised in Table 3.6B. This study was undertaken at S.W.R.S. in 1991 (mean temperature of 22.2^{0}C), with recipient toads collected from the road on the first breeding night of the year. These animals were therefore assumed to have not been exposed to infection since the previous breeding season. All worms transferred had begun feeding on blood whilst developing in their conspecific host. From these transfers, a small percentage of the worms originally transferred were recovered at 3, 7, 16 and 23d p.i. (Table 3.6B).

3.5 Discussion.

Adult *N.scaphiopodis* have previously been reported from pure-bred *S.bombifrons*, *S.multiplicatus* and hybrid individuals (Tinsley, 1982). However, in this study adult and sub-adult forms inhabiting the definitive site were recovered from pure genotypes only (juvenile *N.scaphiopodis* were recovered from all host genotypes). Similar levels of infection were found for all genotypes collected in 1991, except for the prevalence of recent migrants, which may result from differences in emergence. The wider variation in infection levels in 1992 may reflect the combination of reduced sample size and number of collection sites.

The frequency distributions for both respiratory and bladder infections from *S.multiplicatus* correspond to an overdispersed distribution (Fig.3.1). In comparison, the distribution of infection in *S.bombifrons* and hybrid specimens fitted no clear pattern, which is most probably a function of the smaller sample size. In the field, Tinsley & Jackson (1988) reported that 3 consecutive exposures to oncomiracidia led to a 100% prevalence of respiratory infection by *P.americanus*, and the resulting adult population (1 year p.i.) fitted an overdispersed distribution (Tinsley, 1989). The hosts in this study were removed on the first breeding night of the season and the distribution of larval stages examined by the dissection of a random sub-sample. Therefore, the infection levels observed may reflect a combination of the duration of exposure, time of entry into the breeding pond and degree of host mobility (Tinsley, 1989). The levels of adult infection for pure host species are lower than those previously recorded (Tinsley, unpublished), which suggests major temporal fluctuations in recruitment and mortality.

A number of factors influencing the population biology of *P.americanus* have been established (reviewed by Tinsley, 1993) and the close affinity of *P.americanus* to *N.scaphiopodis* suggests that these constraints on reproduction may also exist for the latter species. Within the parasite suprapopulation, a large proportion do not complete reproductive preparation prior to the first opportunity for transmission (Tinsley & Jackson, 1986, 1988; Tocque & Tinsley, 1991a). By the combination of meterological data with laboratory experiments, Tocque & Tinsley (1991b) proposed that temperature may exert a powerful control of the

reproduction of *P.americanus*. In natural and laboratory-controlled populations of *P.americanus*, Tocque & Tinsley (1991a) recorded a positive correlation between worm size and the number of offspring *in utero*. Furthermore, at high burdens, a density-dependent reduction in the rate of offspring accumulation was noted, although burdens of equivalent size were not recorded for *N.scaphiopodis* in this study. In addition *P.americanus*, may also be exposed to the pathological effects of microsporidian hyperparasites, found to infect approximately 11% of the adult population (Cable & Tinsley, 1992a). Cable & Tinsley (1992b) recorded an 11% mortality of migrating worms for artificially stimulated *P.americanus*. Tocque & Tinsley (1994b) suggested that the majority of the decline in infection, from invasion to the next breeding season, resulted from pre-migratory and migratory mortality.

In comparison to females, male *S.multiplicatus* harboured both higher prevalences and intensities of juvenile and sub-adult worms (groups 1 & 2). This may be attributed to their earlier entry into breeding ponds and mobility (Tinsley, 1989). Following infection, Tinsley (1989) found no gender-related differences in the control of the parasite population and concluded that '...the differences in their adult worm burdens can be traced directly to their contrasting levels of invasion'.

There is little information pertaining to the post-oncomiracidial development of anuran polystomatids. In Africa, the neotenic forms of *Polystoma australis*, *P.marmorati* and *P.umthakathi* have been studied (Kok & DuPreez, 1989; Van Niekerk, 1992; Kok, 1990 respectively). In the nearctic, field studies by Tinsley &

Earle (1983) and Tinsley & Jackson (1986) were followed by a comprehensive laboratory assessment of *P.americanus* by Tocque (1990). Understandably, the invasion process and migration thorough the pulmonary system by *N.scaphiopodis* correlates well with the studies of Tinsley & Earle (1983) and Tinsley & Jackson (1986) undertaken at S.W.R.S. rather than the laboratory studies of Tocque (1990). In this study, the extremes of variation for worms making the return migration may reflect sample size, individual heterogeneity or that the first worms to re-enter the mouth were those which entered the lungs in the first few days p.i. followed by those which delayed entry into the lungs.

The commencement of blood-feeding fell within the range described for *P.americanus* by Tinsley & Earle (1983). Blood may not be ingested until 5 days p.i. (also recorded by Tocque, 1990 for *P.americanus*), therefore these worms must initially feed upon host epithelium, perhaps indicated by the opaque nature of the gut. The occasional delay in ingesting blood is of interest as the ultrastructural studies of Allen (1984) suggested that these worms have the capacity to feed on blood immediately after invasion. As noted in the Introduction, the invasion of the lungs by *N.scaphiopodis* and *P.americanus* is a unique feature within the Monogenea (Tinsley & Earle, 1983). Blood vessels are abundant and easily accessible, which is reflected by the change in coloration of the gut. The bright red gut contents, following the first blood meal, quickly become darker and are almost black by the return migration. The bi-product of digestion, haematin, is responsible for the dark pigmentation (Allen, 1984; Tocque & Tinsley, 1992).

It would appear that the principal growth of pre-migratory *N.scaphiopodis* occurs during the blood-feeding period in the lungs (Figs.3.3 & 3.4). However, this does not match the findings of Tinsley & Jackson (1986) in which *P.americanus* doubled in body length during the first 2 week period in the lungs. For total body length, haptor length and pharynx length, indicated that the worms did not grow during the initial migration to the lungs. Although samples sizes were moderate during this period, this phenomenon may have been accentuated by fixation. The major fluctuations at 6, 11, 16 and 24 days p.i. all fell within the standard deviation of the points immediately preceding and following, perhaps reflecting the larger sample sizes at these intervals. Thus sample size and fixation may fully explain the variance recorded. *N.scaphiopodis* recovered from the mouth at 24 days p.i. appeared to be ready to migrate, which is the same order as 26-29 days p.i. migration in field populations of *P.americanus* (Tinsley & Jackson, 1986). This is a period when the host digestive system is at its most active and to protect the worms from degradative enzymes, tegmental vesicles produce a glycocalyx coat prior to migration (Cable & Tinsley, 1992b). Tocque (1990) found that reciprocal exchanges of worms between the respiratory tract and the bladder did not persist, indicating that each form was adapted to its particular habitat.

Estimations of blood-feeding by *P.americanus* inhabiting the definitive site ranged from 0.33µl blood/parasite/week at 2 weeks post migration, increasing with age to a maximum of 5.3µl blood/parasite/week (Tocque & Tinsley, 1992). Tocque (1993) suggested that this drain on host fat reserves represented approximately 7% of annual lipid requirements, which the author proposed could

potentially regulate the host population when the extended periods of starvation were also considered. Tocque & Tinsley (1994a) extended their examination of the detrimental effects of *P.americanus* infection to laboratory controlled populations. The data confirmed the findings of Tocque & Tinsley (1992) and Tocque (1993), with heavily infected individuals typically being in poorer condition than uninfected toads. However, those toads bearing heavy burdens still survived long-term hibernation, suggesting that they would not be unrepresented in the field. Tinsley (1990) stated that although infection may influence host survival, it is inter-related with two other host factors (feeding success and hibernation tolerance).

The development of haptoral sucker pairs occurs simultaneously in *N.scaphiopodis* which concurs with the findings of Thurston (1964) for *Protopolystoma xenopodis*, Salami-Cadoux (1975) for *Eupolystoma alluaudi* and Tocque (1990) for *P.americanus*. This contrasts with the development typically displayed by other polyopisthocotyleans, where the posterior sucker pair develops first and the anterior pair last (Llewellyn, 1963). The variation in haptor length may not only be due to sample size and fixation but also the individual heterogeneity in the development of the definitive haptor. The development of the reproductive system, initially in the form of primordial germ cells, occurs as the definitive haptor is formed (Frankland, 1955; Kearn, 1963; Paul, 1938; Tinsley & Owen, 1975; Tocque, 1990; Wiskin, 1970). The reproductive primordia of *N.scaphiopodis* were observed at 2 days p.i. which correlates with *P.americanus* (Tocque, 1990). However, in this study, the pre-migratory form (Fig.3.6)

possesses three distinct regions, one corresponding to the ovary/vitellaria and two to the testes, as well as the beginnings of the descending limb of the uterus. The arrested worms from the experimental cross-infections at 9 and 12 weeks p.i. confirm that no further development occurs once *N.scaphiopodis* has reached the pre-migratory form.

The worms recovered from both *S.bombifrons* and *S.multiplicatus* are in agreement with the descriptions of *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 by Lamothe-Argumedo (1973a) and Rodgers (1941). The specimens documented in this study, particularly *S.multiplicatus*, exhibited a wider range of sizes than previously recorded (Brooks, 1976; Lamothe-Argumedo, 1973a; Rodgers, 1941). This is most probably due to histological methods and the larger sample size of *S.multiplicatus* rather than characteristic strain differences, which would require further examination of material processed under standardised conditions. In this study, the greater variation was typically found from those worms recovered from *S.multiplicatus* which may again be a function of sample size. In addition to those characters previously described, extra morphological features have been considered (width at the level of the vaginae, HL/L ratio, vitellaria length & width and number of offspring). Following the data presented here, in conjunction with Lamothe-Argumedo (1973a) and Rodgers (1941), the statement by Brooks (1976) that the larval hooklets are '40 to 89μm long by 40-50μm wide at base' must be considered to be erroneous. The overall body proportions of *N.scaphiopodis* are smaller than those reported for *P.americanus* (see Tocque, 1990).

In the original description of *N.scaphiopodis* by Rodgers (1941) the similarity to *Diplorchis ranae* was noted. Murith (1981a) and Tinsley (1983b) stated that, for polystomatids, the elongation of the uterus was correlated with a reduction in medial branching of the gut. Such a pattern is exhibited by *Diplorchis spp.* (see Ozaki, 1935; Lee, 1936), *Metapolystoma cachani* (see Murith, 1981b), *Eupolystoma anterorchis* (see Tinsley, 1978) and *E.alluaudi* (see Combes, Bourgat & Salami-Cadoux, 1973). This is not the case for *N.scaphiopodis*, with the close interdigitation of the digestive tract and uterus (Fig.3.7C) suggested to provide a route for the continuous transfer of nutrients to the developing progeny (Cable & Tinsley, 1991). Furthermore, Cable & Tinsley (1991) reported that nutrients are transferred from the egg capsule lining to the developing embryos in both *N.scaphiopodis* and *P.americanus*, an unprecedented feature in platyhelminth reproductive biology.

The range of sizes recorded by other authors for the marginal hooklets (Table 3.3) is broad when compared to those of other polystomatids (see Murith, 1981a). In this study, only marginal hooklet I was measured from specimens which clearly lay on a plane perpendicular to the objective. This provides accurate documentation of their size and morphology, which is particularly important considering their systematic value in African polystomatids (Murith, 1981a; Kok & Seaman, 1987; Van Niekerk, 1992; Van Niekerk *et al.*, 1992). Applying the analysis described by Murith (1981a) there was no segregation between worms recovered from the two host species in this study (Fig.3.10). There was no variation in gross morphology or size of hooklets from 3 localities or that

portrayed by Rodgers (1941). The remaining measurement, by Lamothe-Argumedo (1973a), appears to be large, which may be the result of geographical variation but perhaps requires reassessment.

Vaucher (1990) proposed a species-specific relationship between total hamulus length and mean blade length/area for South American *Polystoma*. The clear partition between the blade and main shaft of the hamulus of *N.scaphiopodis* facilitated accurate measurements of blade length. On the basis of this analysis there was no separation between worms recovered from *S.bombifrons* and *S.multiplicatus* (Fig.3.9). Although there is no indication of host-specific differences in gross hamulus morphology (Fig.3.8A & B) there does appear to be geographical variation. As noted above, collections of *N.scaphiopodis* from both pure host species, at a number of localities, are required in order to undertake a detailed analysis. Furthermore, in specific populations of the monogenean *Kuhnia scombri* infecting piscine hosts, hamulus length has been correlated to host size (Lakshmi-Perera, 1992) although geographical variation exceeds local variation (Rohde, 1991). The morphology of the hamulus has been a principal tool in the delineation of African anuran polystomatids; however, in a review by Van Niekerk (1992), the author concluded that '...the hamuli cannot serve as a reliable character to distinguish between different *Polystoma* species'.

The differences in morphology between *N.scaphiopodis* and *P.americanus* are further supported by the cross-infection experiments undertaken in this study and by Tinsley (1983a). *S.bombifrons* and *S.multiplicatus* are more closely related to

one another than to *S.couchii*, with a number of authors proposing that they are members of a separate sub-genus (see Chapter 2). The cross-infection of *P.americanus* to *S.bombifrons* and *S.multiplicatus* provides an interesting comparison to the *N.scaphiopodis* exposures. The majority of oncomiracidia did not follow the pattern of invasion described for conspecific exposures by Tinsley & Earle (1983), apparently not receiving the correct stimuli. Moreover, the small number of worms which did enter the respiratory tract appeared to be unable to digest their blood meals, with no darkening noted, indicative of haematin formation. When *P.americanus* was transferred having developed in the lungs of its natural host, a minority could survive to 23 days p.i., but all remained within the respiratory tract. No crosses between *N.scaphiopodis* and *S.couchii* were undertaken; however, Tinsley (1983a) reported these were also unsuccessful. Although there are no records of polystomatid infection of other spadefoot toads in the nearctic, specificity of this nature may assist in the understanding of host phylogeny. The distribution of oncomiracidial tegmental sensilla (chaetotaxy) provides another measure of the phylogenetic relationships within the Polystomatidae. Tinsley (1983a) reported a distinct pattern of 59 tegmental cells for *N.scaphiopodis*, *P.americanus* and *Diplorchis spp.* which was considered to reflect the '...true affinity between Asian and American parasites rather than convergent evolution'.

As noted in the Introduction, the species-specificity proposed for anuran polystomatids in Africa is based primarily on morphological variation (Bourgat, 1977; Combes & Channing, 1978-1979; Euzet, Combes & Knoepffler, 1966; Kok

& van Wyk, 1986; Murith, 1981a) and unpublished cross-infections of larval stages referred to by Bourgat & Salami-Cadoux (1976) Kok & DuPreez (1993). Kennedy (1975) and Murith (1982) suggested that although strict-specificity may exist, it may not be evident in parasite larval stages. However, Combes (1966, 1968) found that the oncomiracidia of European *Polystoma* exhibited a preference when given a choice of host species. This area of polystomatid biology is unclear with some evidence challenging strict host-specificity; for example, Kok & DuPreez (1987) recorded no preference in experimental cross-infections of branchial *Polystoma australis* between two anuran species, or in nature, where these hosts were sympatric. Paradoxically, the same authors have reported other species of *Polystoma* in Africa which do not successfully establish on heterospecific host tadpoles (DuPreez & Kok, 1993). Although specificity does occur, there appear to be a number of exceptions, with the level(s) at which specificity acts remaining unresolved. A full discussion of evidence for specificity relating to the genus *Polystoma* is presented in Chapter 6.

For the infection experiments involving reciprocal crosses of *N.scaphiopodis* between *S.bombifrons* and *S.multiplicatus*, there were a number of variables which could not be eliminated and could only be moderated. For example, although not feasible, a laboratory-reared host population would have provided the ideal recipient group. However, the documented history of the recipient animals provides convincing evidence that the worms recovered must have been the result of experimental infection. The maximum differentiation between any possible pre-existing and experimental infection occurred with those hosts utilised in August-

December 1993. It is questionable whether arrested worms would survive two years in the respiratory tract and/or not migrate during the protracted feeding bouts. Moreover, it is also improbable that all experimental infections will have been lost. Tocque & Tinsley (1994b) found that 25°C was the ideal maintenance temperature for *P.americanus*, with 20% of experimental infections surviving to reach the urinary bladder. It was also noted that, to 6 months post-migration, experimental infections were easily distinguished from natural invasions on the basis of body size and reproductive development (Tocque & Tinsley, 1994b). The variation in the numbers of offspring produced at the same age p.i. may be due to a number of factors: the time of migration, competition (worms may also have been lost prior to dissection and were thus undocumented), host condition (although not correlated in this study) and innate differences in the individual worms themselves. The analysis of invasion success will have been overestimated, as those oncomiracidia remaining on the host skin at the end of the experimental period are unaccounted for. However, if the invasions estimated to have reached the definitive site successfully are of the order of 0.6% this may explain the lower natural infection levels than those recorded by Tinsley (1993) for *P.americanus* infection of *S.couchii*. Tinsley (1993) and Tinsley & Tocque (1994b) commented that there is circumstantial evidence to suggest that host immune responses may play a dominant role in the dramatic reduction in worm burden from infection to the next breeding season.

Murith (1981c) revealed how the reproductive strategy of three African polystomatids were closely adapted to the ecology of each host. The work of

Tinsley and co-workers has elucidated many factors regulating the population dynamics of their nearctic counterparts, which infect almost exclusively terrestrial hosts. Although the majority of this work has been based upon *P.americanus*, the populations of *N.scaphiopodis* may be regulated by the same inter-related factors. Those factors which have been identified are abiotic (temperature), parasite-mediated (age structure, density-dependence, aggregated distribution and pathology) and host-mediated (feeding success, hibernation tolerance, immunological capability and sexual excitement - the stimulus for oncomiracidial release). In addition, the loss of heavily infected hosts from the population will have a disproportionate effect on the parasite suprapopulation, which although undocumented (see Tocque & Tinsley, 1994a) may be a selective force on susceptibility (Tinsley, 1993). Tocque & Tinsley (1994b) commented on the broad annual variation in invasion success and the long term stability of the adult parasite population of *P.americanus* (see Tinsley, 1989, 1993), which indicated that individual heterogeneity in susceptibility to infection may indeed occur. Overall, the low levels of *N.scaphiopodis* infection and a positively skewed frequency distribution will result in the majority of host population suffering minor pathology. As so few hosts of mixed genetic constitution were recovered comparisons regarding hybrid susceptibility were not possible.

In North America, the three genera of anuran polystomatids are each represented by single species, *Polystoma nearcticum* (Paul, 1935) Price, 1939, *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 and *Pseudodiplorchis americanus* (Rodgers & Kuntz, 1940) Yamaguti, 1963. Two of the three species,

N.scaphiopodis (this study) and *P.nearcticum* (refer to Chapter 6), in contradiction to the strict-specificity proposed for anuran polystomatids in Africa, may infect more than one host species.

3.6 References.

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A.

N.scaphiopodis	*S.multiplicatus* (n = 105) prev.	int.	Hybrids (n = 6) prev.	int.	*S.bombifrons* (n = 12) prev.	int.
Group 1	60.0	5.7	50.0	4.7	50.0	7.7
Group 2	2.9	1.3	0	-	8.3	1.0
Group 3	19.0	1.7	0	-	16.7	1.0

B.

N.scaphiopodis	*S.multiplicatus* (n = 17) prev.	int.	Hybrids (n = 1) prev.	int.	*S.bombifrons* (n = 13) prev.	int.
Group 1	58.8	2.5	0	-	23.1	4.3
Group 2	11.8	1.0	0	-	15.4	4.5
Group 3	0	-	0	-	7.7	1.0

Table 3.1 Infection levels of *N.scaphiopodis* recovered from *S.bombifrons*, *S.multiplicatus* and their hybrids collected in the San Simon valley, Arizona/New Mexico, U.S.A. in **A.**) July-August, 1991 and **B.**) July, 1992 (Abbreviations: prev. = prevalence expressed as a percentage; int. = mean intensity).

N.scaphiopodis	Male (n = 73) prev.	int.	Female (n = 32) prev.	int.
Group 1	67.1	5.9	43.8	4.9
Group 2	4.1	1.3	0	-
Group 3	15.1	1.5	28.1	1.9

Table 3.2 Infection levels of *N.scaphiopodis* recovered from male and female *S. ultiplicatus* collec d in the San Simon valley, Arizona/New Mexico, U.S.A. in July-Au ust, 1991 (breviations: as Table 3.1).

	Arizona	Oklahoma	Mexico	Nebraska
B lth	2.21-7.35	2.15-4.35	3.85-5.94	4.45-4.50
B wth	0.80-2.08	0.78-1.78	1.34-1.51	1.47-1.55
W vag	0.54-1.14	-	-	-
Hap lth	0.46-1.44	0.54-0.96	0.92-1.13	1.0
Hap wth	0.76-1.88	0.67-1.23	1.24-1.53	1.34-1.41
O.S. lth	0.07-0.23	0.14-0.22	0.16-0.19	0.16-0.19
O.S. wth	0.13-0.40	0.18-0.28	0.24-0.3	0.25
Ph lth	0.09-0.29	0.11-0.21	0.21-0.22	0.14-0.22
Ph wth	0.07-0.21	0.58-0.17	0.10-0.13	0.13-0.50
T lth	0.34-1.50	-	0.38-0.81	-
T wth	0.13-0.47	-	0.14-0.24	-
Cir lth	13-18μm	-	18μm	-
No. C	8	8	8-9	8
Ov lth	0.13-0.28	0.13-0.17	0.18-0.25	-
Ov wth	0.08-0.18	0.06-0.29	0.13-0.18	-
V lth	0.13-0.24	-	-	-
V wth	0.07-0.19	-	-	-
Larvae	0-89	24-36*	-	-
Embryos	21-291	-	-	-
H.suc dia	0.14-0.32	0.16-0.33	0.27-0.32	0.27-0.28
Ham lth	236-482μm	176-406μm	418-434μm	406μm
Hook lth	26-28μm	18-28.5μm	30-33μm	40-89μm

Table 3.3 Body dimensions of sexually mature, adult *N.scaphiopodis* from 4 geographical locations. Measurements in mm (except where indicated) and represented as the range (in accordance with previous descriptions). Arizona, n = 73, Host: *S.multiplicatus*, this study; Oklahoma, n = 45, Host: *S.bombifrons*, Rodgers (1941); Mexico, n = 50+, Host: *S.multiplicatus*, Lamothe-Argumedo (1973a ; Nebraska, n = ?, Host: *S.bombifrons*, Brooks (1976).

Abbreviations: B lth, Body length; **B wth**, Maximum width; **W vag**, Width at the level of the vaginae; **Hap lth**, Haptor length; **Hap wth**, Haptor width; **O.S. lth**, Oral sucker length; **O.S. wth**, Oral sucker width; **Ph lth**, Pharynx length; **Ph wth**, Pharynx width; **T lth**, Testes length; **T wth**, Testes width; **C lth**, Cirrus spine length; **No. C**, Number of cirrus spines; **Ov lth**, Ovary length; **Ov wth**, Ovary width; **V lth**, Vitellaria length; **V wth**, Vitellaria width; **Larvae**, Number of encapsulated oncomiracidia *in utero* (possessing eyespots and cilia); **Embryos**, Number of developing embryos *in utero* (without eyespots and cilia); **H.suc dia**, Haptoral sucker diameter; **Ham lth**, Hamulus length, **Hook lth**, Marginal hooklet I length; *, '2 to 3 dozen' Rodgers (1941).

	A.			B.		
	Mean	S.D.	Range	Mean	S.D.	Range
B lth	4.88	1.28	2.21-7.35	5.62	0.84	3.16-6.48
B wth	1.31	0.31	0.80-2.08	1.61	0.25	0.78-1.96
W vag	0.78	0.12	0.54-1.14	0.80	0.09	0.62-0.92
Hap lth	0.95	0.21	0.46-1.44	1.19	0.19	0.76-1.48
Hap wth	1.36	0.27	0.76-1.88	1.65	0.22	0.98-1.92
O.S. lth	0.14	0.03	0.07-0.23	0.15	0.02	0.10-0.20
O.S. wth	0.28	0.04	0.13-0.40	0.28	0.02	0.24-0.31
Ph lth	0.15	0.03	0.09-0.30	0.17	0.01	0.15-0.20
Ph wth	0.11	0.02	0.07-0.21	0.12	0.01	0.08-0.14
T lth	0.71	0.25	0.34-1.50	0.87	0.24	0.38-1.39
T wth	0.29	0.07	0.13-0.47	0.25	0.05	0.18-0.35
Cir lth	15µm	1µm	13-18µm	16µm	1µm	15-17µm
No. C	8	-	-	8	-	-
Ov lth	0.20	0.04	0.13-0.28	0.22	0.03	0.18-0.27
Ov wth	0.14	0.02	0.08-0.18	0.14	0.02	0.11-0.18
V lth	0.17	0.03	0.13-0.24	0.20	0.03	0.14-0.22
V wth	0.11	0.03	0.07-0.19	0.10	0.03	0.07-0.14
Larvae	13.6	22.2	0-89	38.8	26.3	0-69
Embryos	105.8	57.3	21-291	104.1	40.3	10-174
H.suc dia	0.24	0.04	0.14-0.32	0.29	0.04	0.19-0.36
Ham lth	386µm	60µm	236-482µm	414µm	41µm	306-456µm
Hook lth	27µm	1µm	26-28µm	28µm	1µm	27-29µm

Table 3.4 Body dimensions of sexually mature *N.scaphiopodis* recovered from the urinary bladder of **A.)** *Scaphiopus multiplicatus* (n = 73) and **B.)** *S.bombifrons* (n = 22) collected in the San Simon valley, Arizona/New Mexico, U.S.A.

Table 3.5A.

#	Host	Status	Exp	Suc	Duration	Pre-inf	Recov
1	*S.m.*	Cross-inf	140	-	1 day	-	31
2	*S.b.*	Control	140	-	1 day	-	60
3	*S.b.*	Cross-inf	34	-	1 day	-	5
4	*S.m.*	Control	34	-	1 day	-	3
5	*S.b.*	Cross-inf	75	68%	1.5 days	-	32
6	*S.m.*	Control	75	65%	1.5 days	+	15
7	*S.m.*	Cross-inf	72	-	2 days	-	0
8	*S.m.*	Cross-inf	95	58%	3 days	-	23
9	*S.m.*	Cross-inf	60	-	4 days	-	2
10	*S.m.*	Cross-inf	40	75%	3 wks	-	4
11	*S.m.*	Cross-inf	40	75%	3 wks	-	1
12	*S.m.*	Cross-inf	40	85%	3 wks	-	4
13	*S.b.*	Control	40	60%	3 wks	-	8
14	*S.b.*	Cross-inf	60	-	3 wks	-	37
15	*S.m.*	Control	60	-	3 wks	-	0
16	*S.m.*	Control	56	-	3 wks	-	0
17	*S.m.*	Cross-inf	50	66%	7 wks	-	0
18	*S.m.*	Cross-inf	50	90%	7 wks	-	Died
19	*S.b.*	Control	50	92%	7 wks	-	0
20	*S.b.*	Cross-inf	50	88%	7 wks	-	1
21	*S.m.*	Control	50	96%	7 wks	-	Died
22	*S.b.*	Cross-inf	100	66%	7 wks	-	0
23	*S.b.*	Cross-inf	100	20%	7 wks	-	0
24	*S.m.*	Control	83	83%	7 wks	-	0
25	*S.m.*	Cross-inf	100	86%	8 wks	-	0
26	*S.m.*	Cross-inf	100	78%	8 wks	-	0
27	*S.m.*	Cross-inf	100	13%	8 wks	-	0
28	*S.b.*	Control	100	65%	8 wks	-	Died
29	*S.b.*	Cross-inf	30	-	9 wks	+	0
30	*S.b.*	Cross-inf	30	70%	9 wks	-	1[a]
31	*S.m.*	Control	30	90%	9 wks	-	1[a]

Table 3.5A.

#	Host	Status	Exp	Suc	Duration	Pre-inf	Recov
32	*S.m.*	Cross-inf	80	98%	12 wks	-	0
33	*S.m.*	Cross-inf	80	85%	12 wks	-	0
34	*S.b.*	Control	80	88%	12 wks	-	0
35	*S.m.*	Cross-inf	100	26%	12 wks	-	0
36	*S.m.*	Cross-inf	100	68%	12 wks	-	1+1*
37	*S.m.*	Cross-inf	100	62%	12 wks	+	0
38	*S.m.*	Cross-inf	100	94%	13.5 wks	-	0
39	*S.m.*	Cross-inf	100	71%	13.5 wks	+	0
40	*S.m.*	Cross-inf	100	85%	13.5 wks	-	1
41	*S.b.*	Control	100	82%	13.5 wks	-	0
42	*S.m.*	Cross-inf	100	96%	14 wks	-	0
43	*S.b.*	Control	100	95%	14 wks	-	4
44	*S.b.*	Cross-inf	60	77%	15 wks	+	7
45	*S.m.*	Cross-inf	85	93%	15 wks	-	0
46	*S.m.*	Cross-inf	85	98%	15 wks	-	0
47	*S.m.*	Cross-inf	100	81%	15 wks	-	1
48	*S.m.*	Cross-inf	100	94%	15 wks	-	0
49	*S.m.*	Cross-inf	100	69%	15 wks	-	0
50	*S.b.*	Control	100	67%	15 wks	-	0
51	*S.b.*	Cross-inf	100	69%	15 wks	-	0
52	*S.m.*	Control	100	51%	15 wks	-	1
53	*S.m.*	Cross-inf	100	72%	15 wks	-	0
54	*S.m.*	Cross-inf	100	74%	15 wks	+	0
55	*S.m.*	Cross-inf	100	75%	15 wks	-	5
56	*S.m.*	Cross-inf	100	71%	15 wks	-	1*
57	*S.m.*	Cross-inf	100	88%	15 wks	-	1
58	*S.m.*	Cross-inf	100	90%	15 wks	-	Died
59	*S.m.*	Cross-inf	100	76%	15 wks	-	Died
60	F1	Cross-inf	100	77%	15 wks	-	Died
61	F1	Cross-inf	100	79%	15 wks	-	0
62	F1	Cross-inf	100	94%	15 wks	-	Died

Table 3.5A.

#	Host	Status	Exp	Suc	Duration	Pre-inf	Recov
63	BKM	Cross-inf	100	76%	15 wks	-	0
64	*S.m.*	Control	100	67%	15 wks	-	Died
65	*S.m.*	Control	100	91%	15 wks	-	13
66	*S.m.*	Cross-inf	85	79%	15 wks	-	0
67	*S.m.*	Cross-inf	85	83%	15 wks	-	0
68	*S.m.*	Cross-inf	100	92%	16 wks	-	3
69	*S.m.*	Cross-inf	50	98%	16 wks	-	0
70	*S.m.*	Cross-inf	50	96%	16 wks	-	1
71	*S.b.*	Control	100	91%	16 wks	+	0
72	BKM	Cross-inf	100	95%	16 wks	-	Died
73	*S.m.*	Control	50	98%	16 wks	+	0
74	*S.b.*	Cross-inf	100	86%	16 wks	+	2
75	*S.m.*	Control	100	89%	16 wks	-	1
76	*S.m.*	Control	50	90%	16 wks	-	0
77	*S.b.*	Cross-inf	100	41%	17 wks	-	0
78	*S.b.*	Cross-inf	100	60%	17 wks	-	0
79	*S.b.*	Cross-inf	100	63%	17 wks	-	0
80	*S.m.*	Control	100	64%	17 wks	-	0
81	*S.m.*	Cross-inf	130	98%	17 wks	-	0
82	*S.m.*	Cross-inf	130	90%	17 wks	-	1
83	*S.b.*	Control	130	92%	17 wks	-	Died
84	*S.m.*	Cross-inf	100	89%	20 wks	-	1
85	*S.b.*	Control	100	89%	20 wks	-	0
86	*S.m.*	Cross-inf	100	94%	20 wks	-	1
87	*S.b.*	Control	100	88%	20 wks	-	Died
88	*S.m.*	Cross-inf	100	93%	21 wks	-	0
89	*S.m.*	Cross-inf	100	47%	21 wks	-	Died
90	*S.b.*	Control	100	76%	21 wks	-	0
91	*S.m.*	Cross-inf	100	78%	21 wks	-	0
92	*S.m.*	Cross-inf	100	89%	21 wks	-	0
93	*S.m.*	Cross-inf	100	80%	21 wks	-	0

A.

#	Host	Status	Exp	Suc	Duration	Pre-inf	Recov
94	*S.b.*	Control	100	93%	21 wks	-	0
95	*S.b.*	Control	100	94%	21 wks	-	0
96	*S.m.*	Control	50	80%	25 wks	-	13
97	*S.b.*	Cross-inf	25	64%	26 wks	-	0
98	*S.m.*	Control	25	28%	26 wks	-	2
99	*S.b.*	Cross-inf	70	86%	26 wks	-	0
100	*S.m.*	Control	70	54%	26 wks	-	1[b]

B.

#	Host	Status	Exp	Suc	Duration	Pre-inf	Recov
1	*S.b.*	Cross-inf	159	-	1 wk	-	67
	S.m.	Control				-	0
2	*S.b.*	Cross-inf	44	-	2 wks	-	7
	S.m.	Control				-	0
3	*S.b.*	Cross-inf	80	-	3 wks	-	14
	S.m.	Control				-	23
4	*S.m.*	Cross-inf	55	96%	8 wks	-	0
	S.b.	Control				-	0

Table 3.5 Cross-infection experiments involving **A.)** single exposures and **B.)** multiple exposures of *S.bombifrons* and *S.multiplicatus* to oncomiracidia of *N.scaphiopodis* from pre-typed donors.

Abbreviations: **#**, experiment number; **Status**, infection type; **Exp**, number of oncomiracidia utilised; **Suc**, estimated percentage of successful invasions; **Duration**, experimental period; **Pre-inf**, pre-existing infection by *N.scaphiopodis*; **Recov**, number of worms recovered from experimental exposure; ***S.b.***, *S.bombifrons*; ***S.m.***, *S.multiplicatus*; **Died**, host died prior to completion of the experimental period; wks, weeks; [a], experimental infection recovered from the respiratory tract; [b], recently dead *N.scaphiopodis* recovered from the urinary bladder;

A.

Host	No. of Oncomiracidia	Exposure period/hours	Infection period/days	No. of worms recovered
S.m.	125	1	1	6
S.b.	125	1	1	5
S.m.	100	2	2	3
S.b.	160	2	2	9
S.b.	160	2	3	7
S.m.	140	2	4	0
S.b.	129	2	4	1*
S.m.	140	2	5	0
S.b.	140	2	5	0
S.m.	125	2	6	3
S.b.	125	2	7	1
S.m.	180	2	10	0
S.b.	180	2	10	0
S.m.	100	1	14	0
S.b.	180	2	14	0

B.

Host	No. of worms transferred	Infection period/days	No. of worms recovered
S.m.	4	3	1
S.m.	41	7	6
S.m.	33	16	3
S.m.	28	23	0
S.m.	33	23	0
S.m.	33	23	2

Table 3.6 Cross-infection experiments involving **A.**) the exposure of *S.bombifrons* and *S.multiplicatus* to oncomiracidia of *P.americanus* in the laboratory and **B.**) the transfer of respiratory worms *P.americanus* to the oral cavity of *S.multiplicatus* in the field. (Abbreviations: *S.b.* = *S.bombifrons*; *S.m.* = *S.multiplicatus*; * = recently dead).

3.7 Legends.

Fig.3.1 Frequency distribution of *N.scaphiopodis* recovered from **A.)** the respiratory tract and **B.)** the urinary bladder of *S.bombifrons* (n = 12), F_1 hybrids (n = 6) and *S.multiplicatus* (n = 105) collected in July/August 1991 from the San Simon valley, Arizona/New Mexico, U.S.A.

Fig.3.2 Distribution of post-oncomiracidial *N.scaphiopodis* in **A.)** the nasal passages, **B.)** oral cavity (including the vocal sac of males) and **C.)** lungs from 1-24 days p.i. from dissections at S.W.R.S. in 1991/1992. Sample size of both hosts and helminths displayed.

Fig.3.3 Relationship between total body length and haptor length during the development of post-oncomiracidial *N.scaphiopodis* 1-24 days p.i. from dissections at S.W.R.S. in 1991/1992.

Fig.3.4 Pharynx length of post-oncomiracidial *N.scaphiopodis* 1-24 days p.i. from dissections at S.W.R.S. in 1991/1992.

Fig.3.5 Hamulus development of post-oncomiracidial *N.scaphiopodis* at **A.)** 2 days, **B.)** 4 days, **C.)** 8 days, **D.)** 10 days, **E.)** 14 days, **F.)** 16 days, **G.)** 19 days and **H.)** 24 days p.i. **Scale bar:** 0.1mm.

Fig.3.6 *N.scaphiopodis*, pre-migratory form at 24 days p.i. Abbreviations: **c**, cirrus; **gc** & ▓, gut caecum; **ham**, hamulus; **hk**, marginal hooklet; **os**, oral sucker; **ov** & ▒, developing ovary; **ph**, pharynx; **suc**, haptoral sucker; **ts** & ▒, developing testis; **ut**, developing uterus. **Scale bar:** 0.2mm.

Fig.3.7 *N.scaphiopodis*, sexually mature, gravid adult. **A.)** Dorsal view displaying the intestinal outline ▒ and uterus, with the positions of ovary ▒ and testes indicated ▓, **B.)** Reproductive system (omitting the Mehlis'gland and progeny) and **C.)** Cross-section at the level indicated (**X**) in **A.**, demonstrating the close association of the gut and uterus. Abbreviations: **c**, cirrus; **e**, developing embryo; **gc** & ▒, gut caecum; **gi**, genito-intestinal canal; **ham**, hamulus; **l**, fully developed larva; **o**, oviduct; **ol**, ovovitelline canal; **os**, oral sucker; **ov** & ▒, ovary; **ph**, pharynx; **suc**, haptoral sucker; **ts** & ▓, testis; **ut**, uterus, **v**, vitellaria; **vag**, vaginae; **vd**, vas deferens; **ve**, vas efferens. **Scale bar:** 0.2mm.

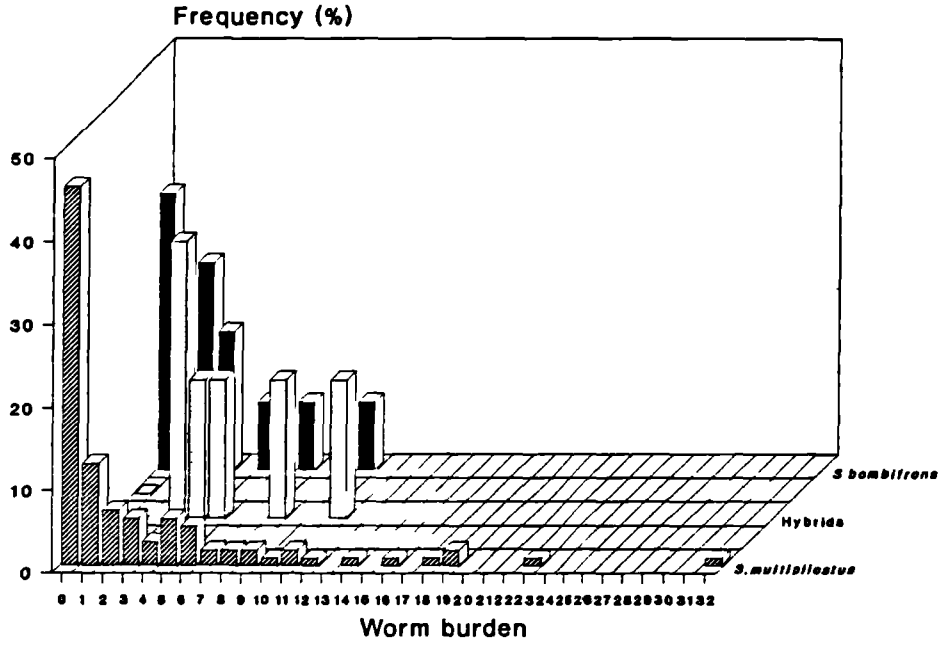
Fig.3.8 Geographical variation of *N.scaphiopodis* hamulus morphology. Specimens recovered from 4 U.S. States and Mexico: **A.)** Host - *S.multiplicatus*, Arizona; **B.)** Host - *S.bombifrons*, Arizona; **C.)** Host - *S.bombifrons*, Texas; **D.)** Host - *S.bombifrons*, Nebraska, reproduced from Brooks (1976), **E.)** Host - *S.multiplicatus*, Mexico, reproduced from Lamothe-Argumedo (1973a) and **F.)** Host - *S.bombifrons*, Oklahoma reproduced from Rodgers (1941). **Scale bars:** 0.1mm.

Fig.3.9 Scatter diagram of total hamulus length and mean blade length for *N.scaphiopodis* recovered from *S.bombifrons* (⊡) and *S.multiplicatus* (+).

Fig.3.10 Scatter diagram of marginal hooklet I length (b) and length to the bifurcation of the guard (a) for *N.scaphiopodis* recovered from *S.bombifrons* (⊡) and *S.multiplicatus* (+).

Fig.3.11 Laboratory temperature and feeding regime for all non-experimental toads.

A


Frequency (%)
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40
30
20
10
0
S bombifrons
Hybrids
S.multiplicatus
Worm burden

B

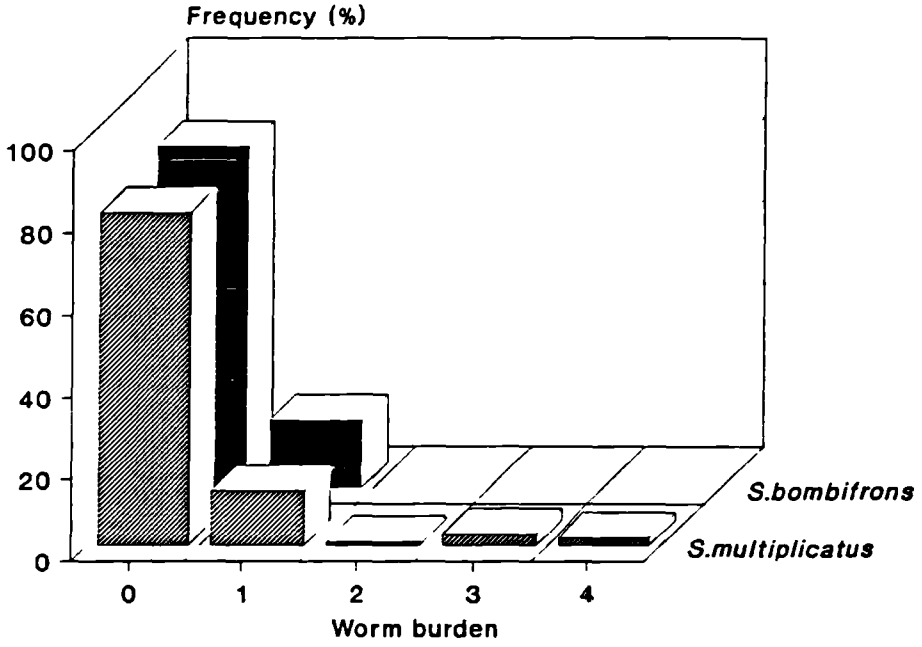
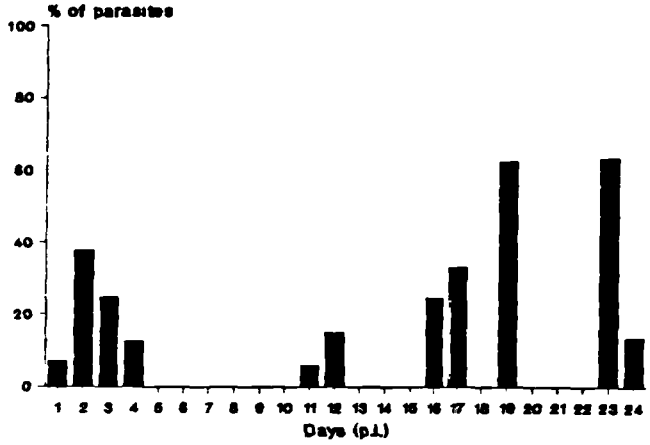
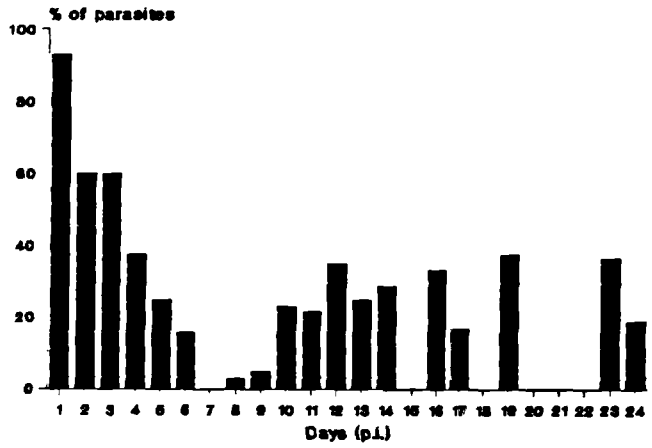

Frequency (%)
100
80
60
40
20
0
S.bombifrons
S.multiplicatus
0
1
2
3
4
Worm burden

Fig.3.1

A



B



C

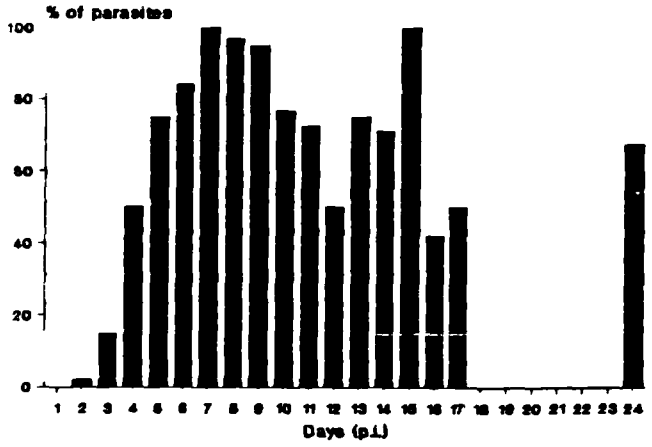


Fig.3.2

helminths	14	46	20	8	4	19	8	36	20	26	61	20	8	14	4	67	8	8	16	-	-	-	11	38
hosts	6	9	14	10	2	4	3	12	8	9	7	12	6	7	2	22	2	4	6	-	-	-	9	2

Fig.3.3

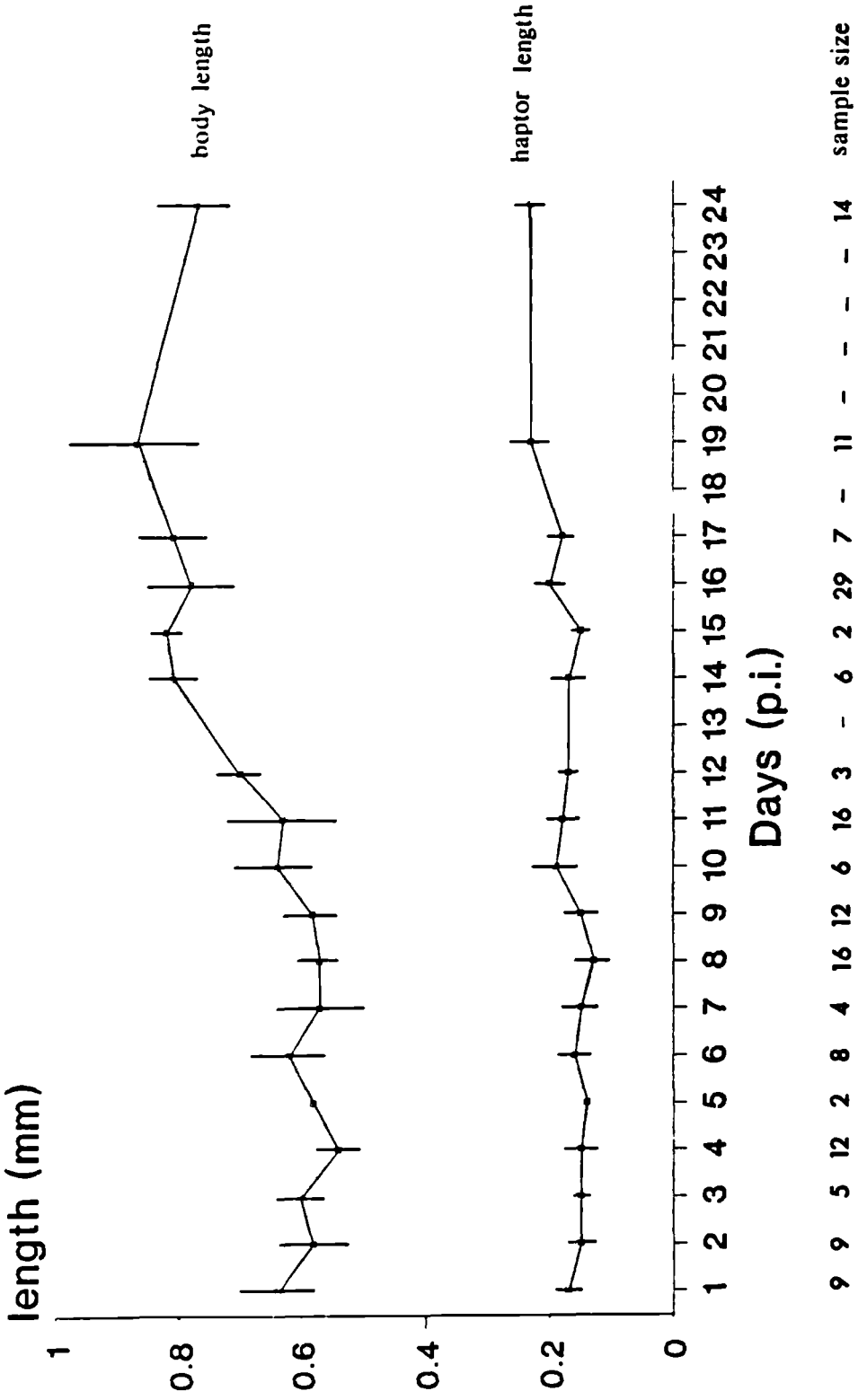
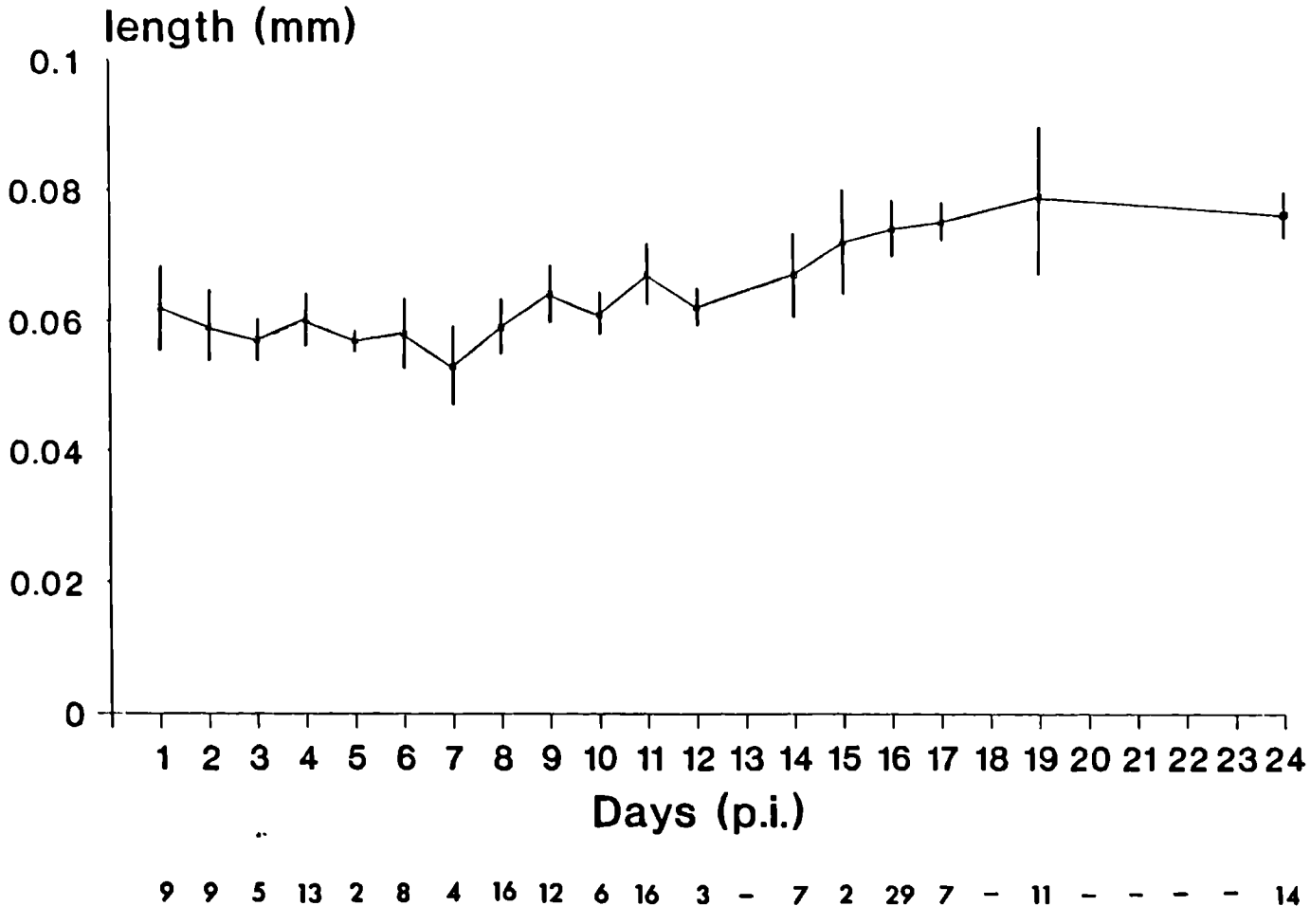

length (mm)
1
0.8
0.6
0.4
0.2
0
body length
haptor length
Days (p.i.)
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
sample size
9 9 5 12 2 8 4 16 12 6 16 3 - 6 2 29 7 - 11 - - - - - 14

Fig.3.4


length (mm)
0.1
0.08
0.06
0.04
0.02
0
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
Days (p.i.)
9 9 5 13 2 8 4 16 12 6 16 3 - 7 2 29 7 - 11 - - - - 14
sample size

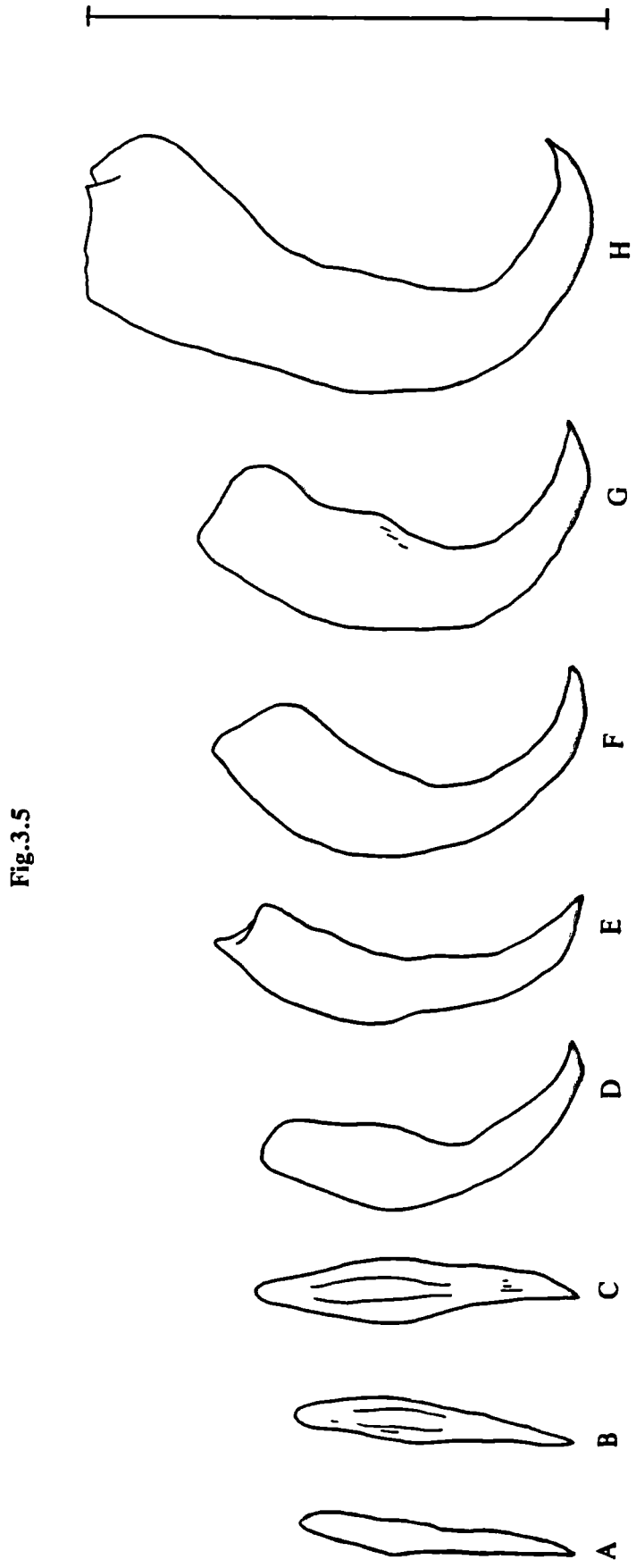


Fig.3.5

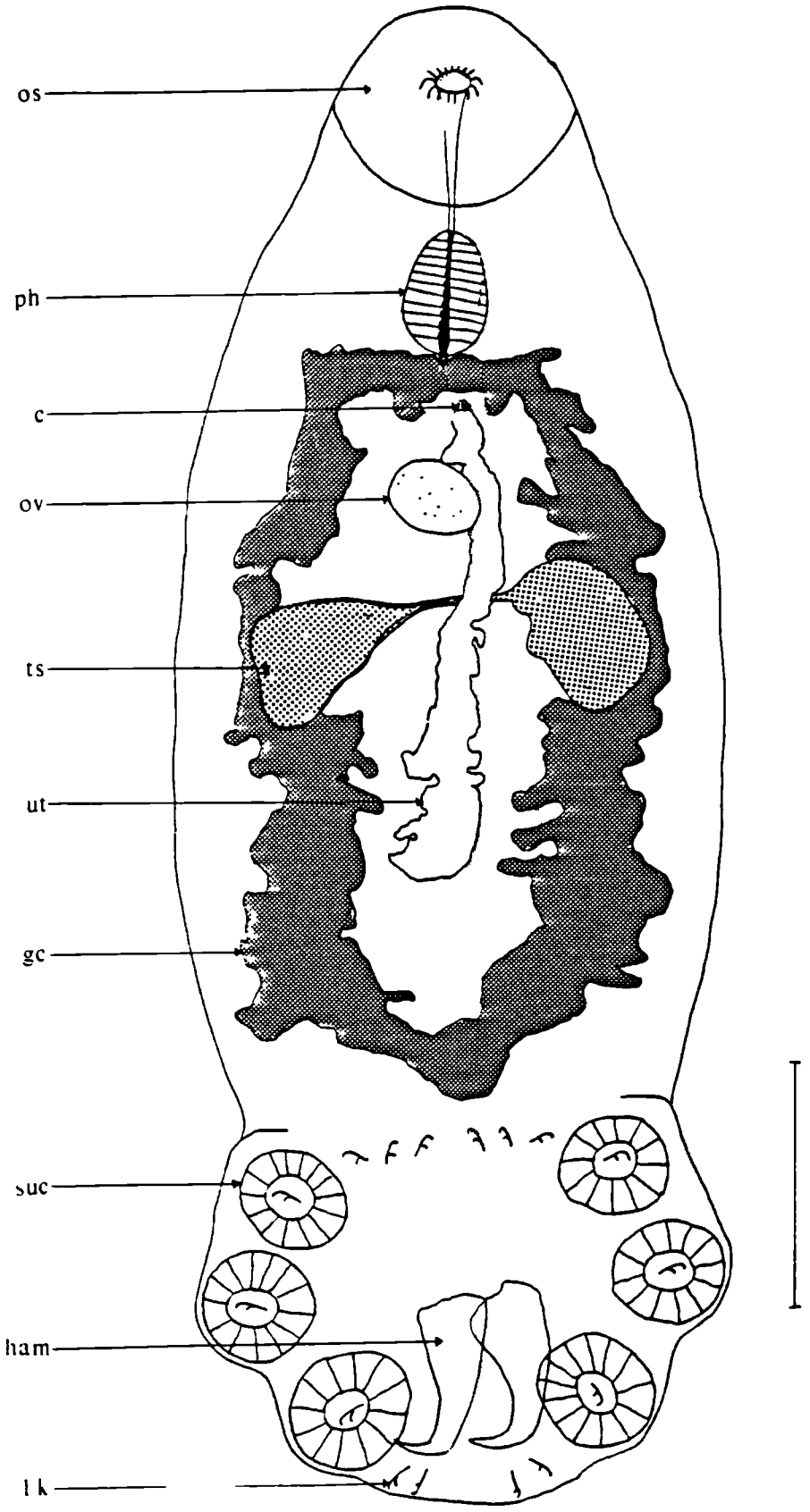


Fig.3.6

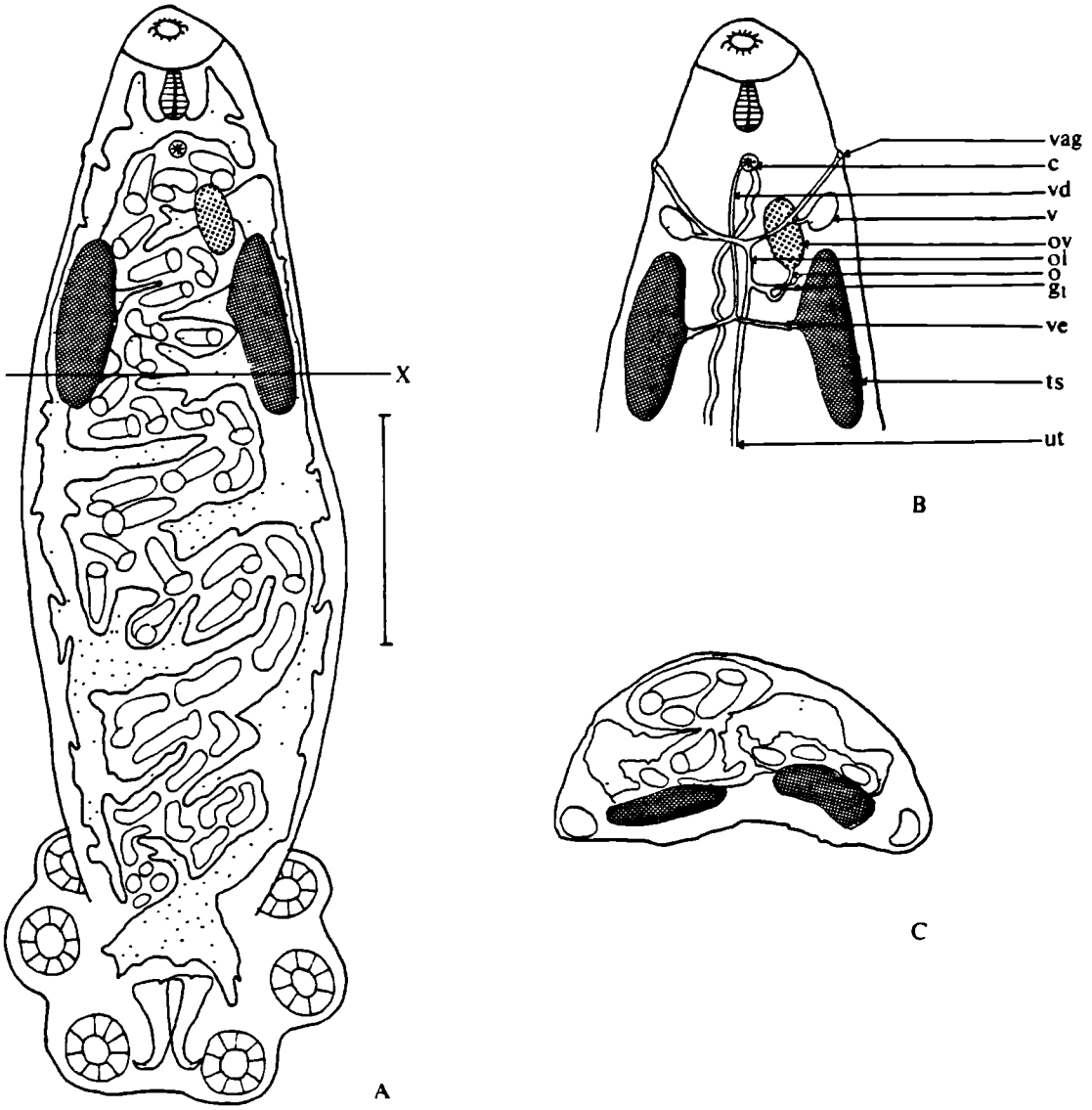


Fig.3.7

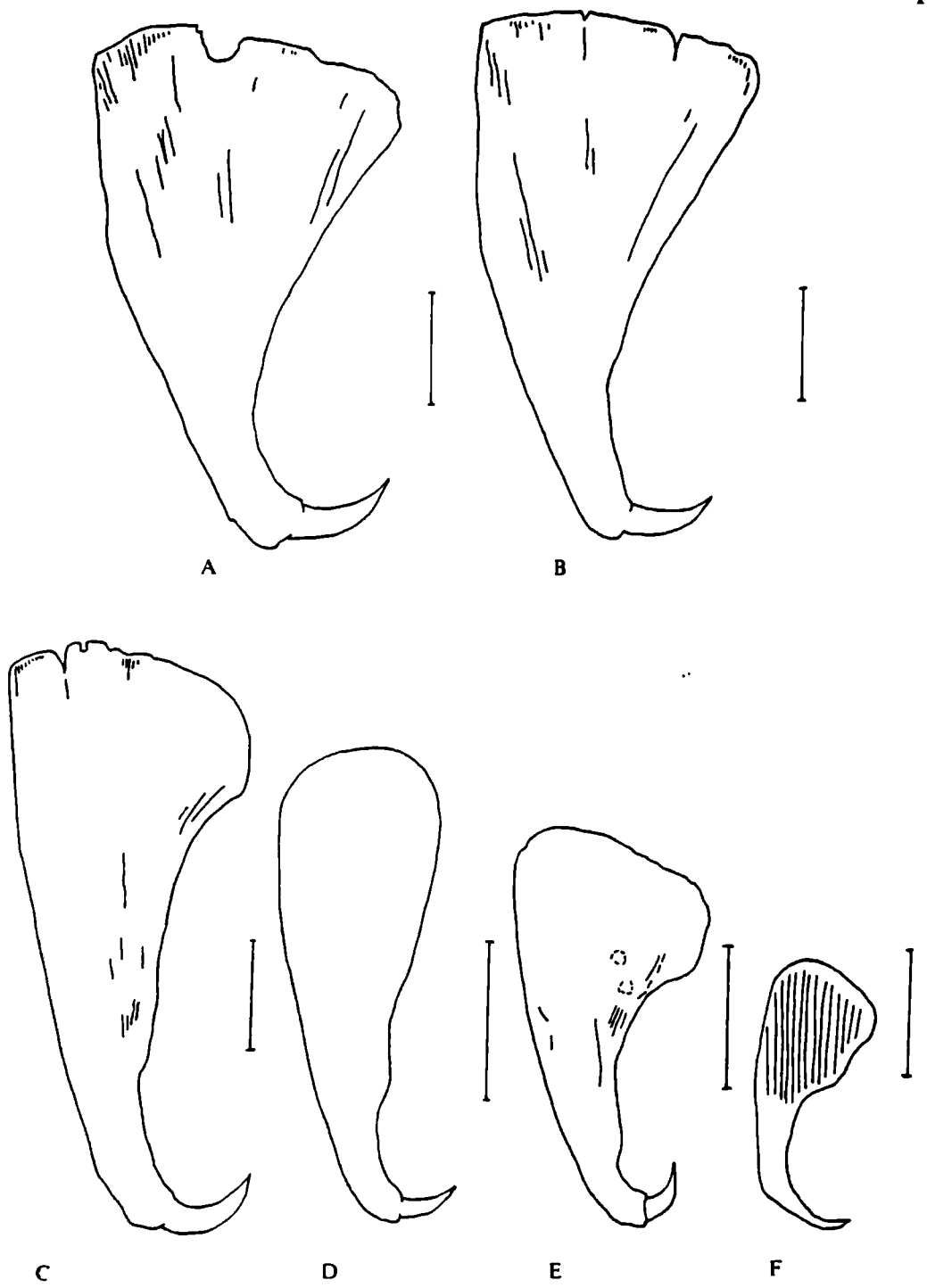


Fig.3.8

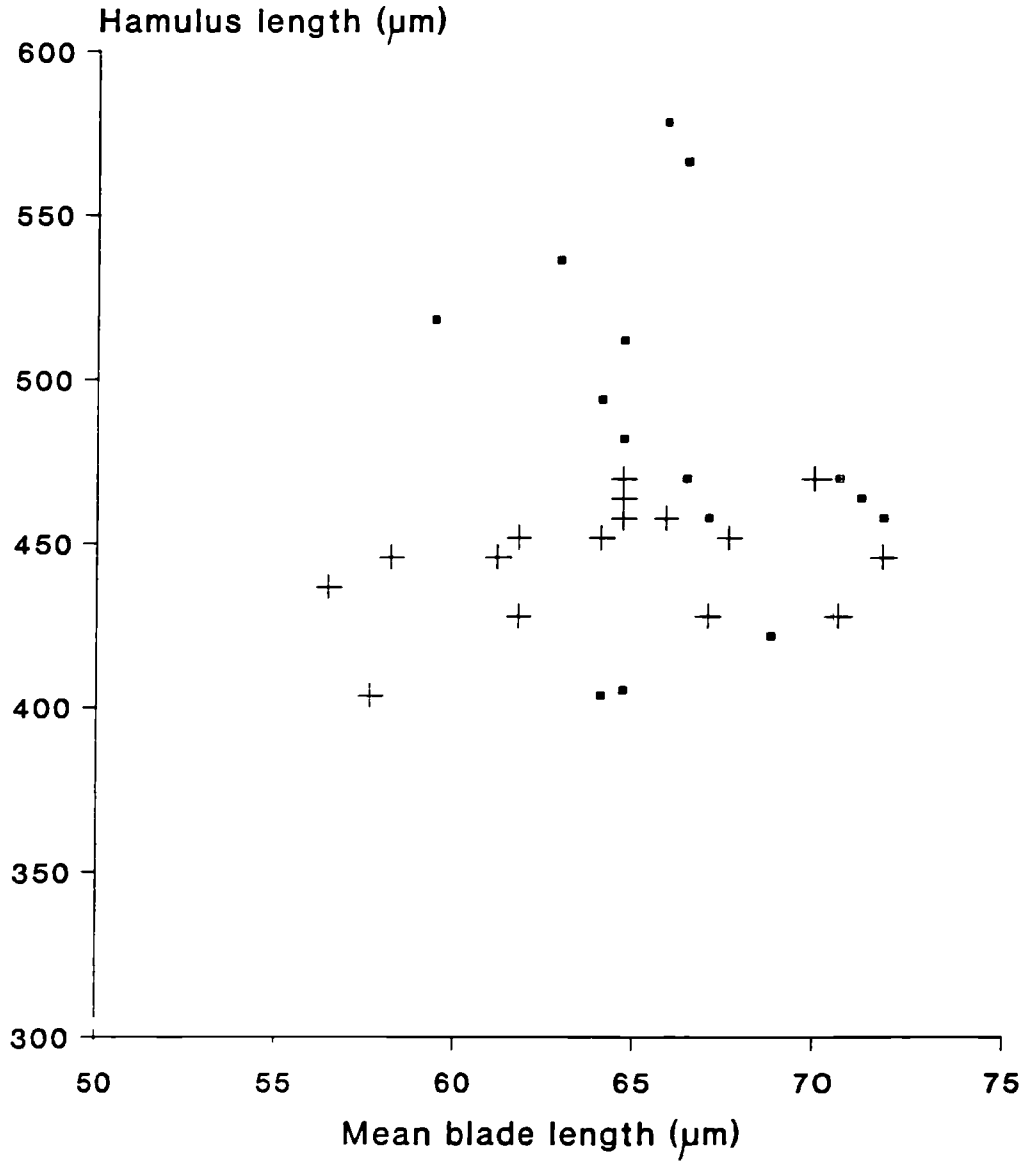

Hamulus length (µm)
600
550
500
450
400
350
300
50
55
60
65
70
75
Mean blade length (µm)

Fig.3.9

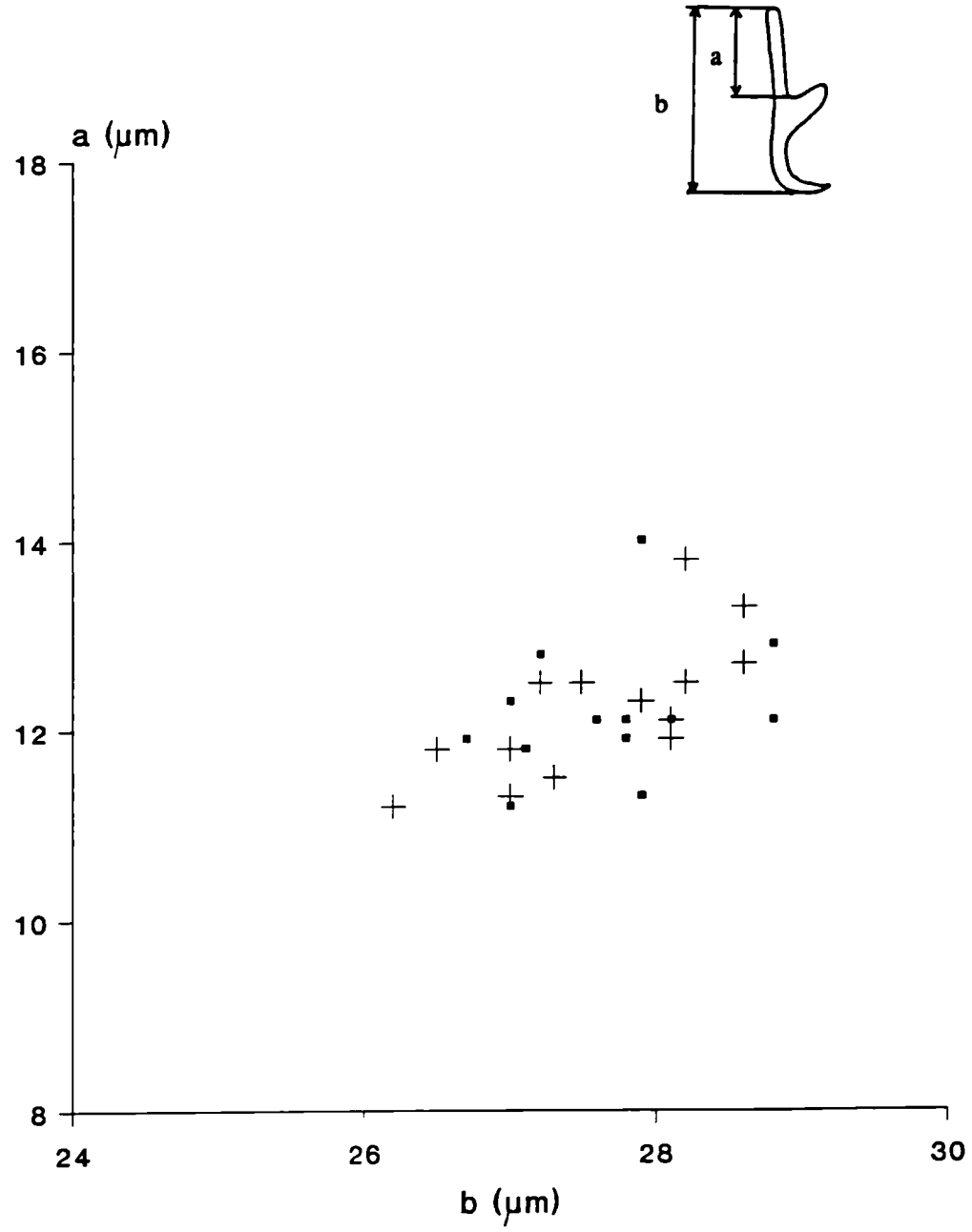


Fig.3.10

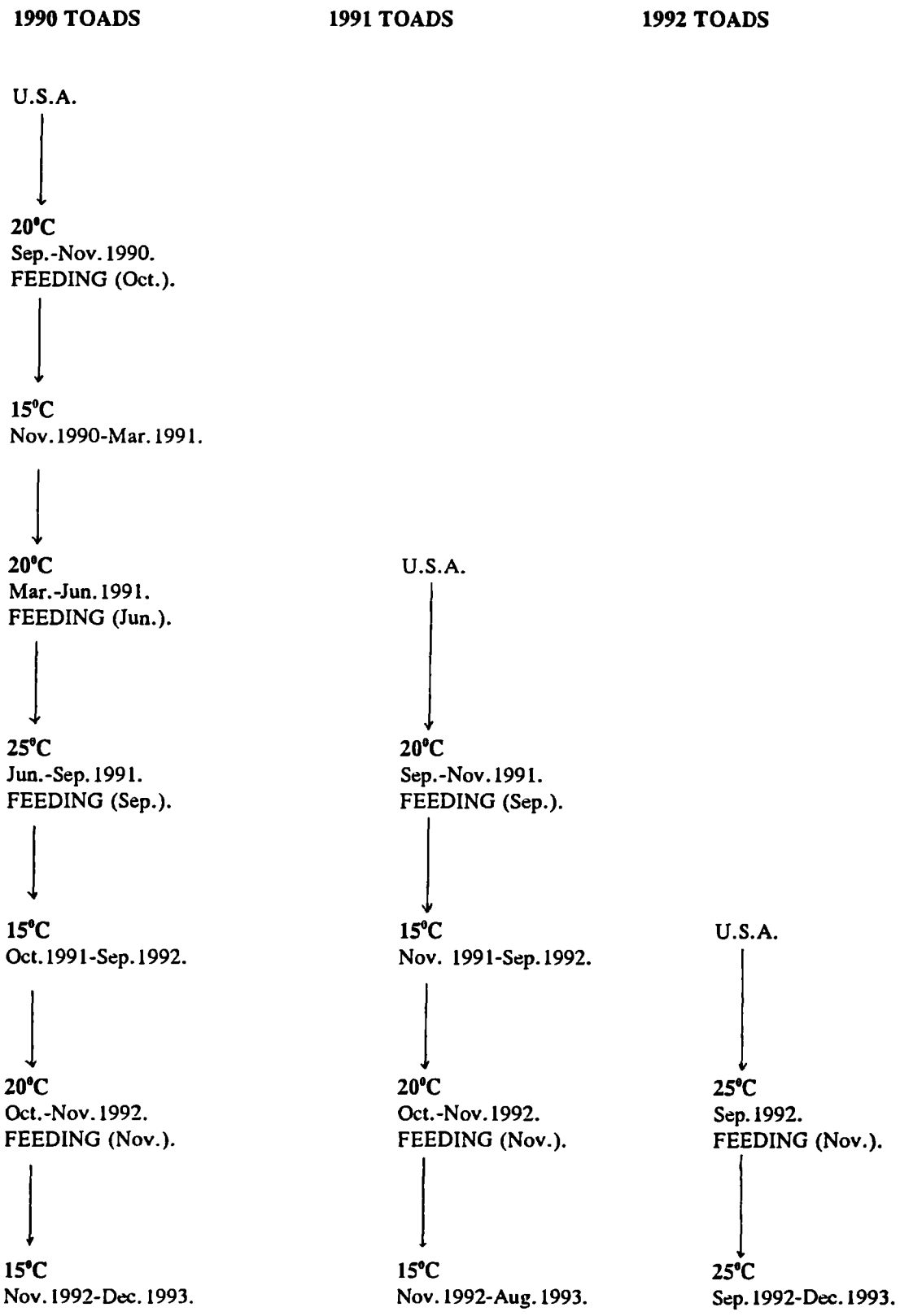

1990 TOADS
1991 TOADS
1992 TOADS
U.S.A.
20°C
Sep.-Nov. 1990.
FEEDING (Oct.).
15°C
Nov. 1990-Mar. 1991.
20°C
Mar.-Jun. 1991.
FEEDING (Jun.).
25°C
Jun.-Sep. 1991.
FEEDING (Sep.).
15°C
Oct. 1991-Sep. 1992.
20°C
Oct.-Nov. 1992.
FEEDING (Nov.).
15°C
Nov. 1992-Dec. 1993.
U.S.A.
20°C
Sep.-Nov. 1991.
FEEDING (Sep.).
15°C
Nov. 1991-Sep. 1992.
20°C
Oct.-Nov. 1992.
FEEDING (Nov.).
15°C
Nov. 1992-Aug. 1993.
U.S.A.
25°C
Sep. 1992.
FEEDING (Nov.).
25°C
Sep. 1992-Dec. 1993.

Fig.3.11

Chapter 4.

A parasitological survey of the Spadefoot toads *Scaphiopus bombifrons*, *S.multiplicatus* (Anura:Pelobatidae) and their hybrids from the San Simon valley, southern Arizona/New Mexico, U.S.A.

4.1 Abstract.

The spadefoot toads, *Scaphiopus bombifrons* and *S.multiplicatus* are sympatric in the San Simon valley, which runs along the borders of S.E. Arizona and S.W. New Mexico, U.S.A. Reproductive isolation is maintained by female choice, based on male call characteristics. However, in the rapid and torrential summer monsoons, common to this region, scramble competition by males may lead to a breakdown in these isolating mechanisms, resulting in heterospecific crosses.

A full parasitological survey of 154 spadefoot toads was carried out over two years. This revealed that a common, restricted fauna of parasites occurs in both pure and hybrid genotypes. Representatives of five parasitic groups were found: *Neodiplorchis scaphiopodis* (Monogenea); *Clinostomum complanatum* (Digenea); *Distoichometra bufonis* (Cestoda); *Aplectana itzocamensis*, *Physaloptera sp.*, unidentified encysted larvae and microfilariae (Nematoda); *Nyctotherus sp./Balantidium sp.* and *Opalina sp.* (Protozoa). No significant relationship was found between parasitic infection and a suite of host factors, except for the larval nematode, *Physaloptera sp.* In this case, regression analysis revealed a significant positive correlation between host snout-vent length (SVL) and physalopterid infection ($R^2 = 6.1$, $F = 6.02$, $p = 0.016$).

A number of new host records and a new locality record for the State of Arizona are described. The diversity of the parasite fauna is linked to host ecology and genetics. A checklist of all recorded parasites of *Scaphiopus spp.* is included.

4.2 Introduction.

This study was based in the San Simon valley of southeastern Arizona and southwestern New Mexico, U.S.A. The vegetation is Chihuahuan desertscrub (formed of creosote, mesquite and ocotillo) and desert grassland (Lowe, 1964). The elevation of the study area ranges from 1520m at Portal, Arizona to 1280m on the valley floor at Rodeo, New Mexico (Dimmitt & Ruibal, 1980a). Approximately half of the average rainfall (225mm) falls in monsoonal rains during July to September (Ruibal, Tevis & Roig, 1969). These summer monsoons are created from warm, moist air, moving over the mountainous terrain, resulting in convection storms.

Spadefoot toads, which are common in the area, have been the subject of a number of ecological, evolutionary and physiological examinations (see Duellman & Trueb, 1986). The geographical ranges of *Scaphiopus bombifrons* and *S.multiplicatus* overlap in a number of States of the U.S.A. and Mexico (Brown, 1976; Sattler, 1985; Stebbins, 1985) and as described in Chapter 2, a dynamic hybrid zone between *S.bombifrons* and *S.multiplicatus* exists in this region.

S.bombifrons is generally considered to be a plains species (Stebbins, 1985), inhabiting lower elevation grasslands and river valleys (Tanner, 1989; Wasserman, 1964). *S.multiplicatus* favours the desert scrub at higher elevations, being less abundant in grassland areas (Ruibal *et al.*, 1969; Tanner, 1989). Simovich (1985) noted that *S.multiplicatus* is the more abundant species of the two in the San Simon valley, and postulated that the irregular distribution of *S.bombifrons* is

related to soil preference.

S.multiplicatus burrows in the soil, to depths of 90cm, for approximately 9 months of the year. It has been suggested that digging sites are chosen and not randomly selected (Simovich, 1985). By maintaining an osmotic potential equivalent to the surrounding soil, moisture loss is prevented (Ruibal *et al.*, 1969). This is achieved by storage of urea in the body fluids (up to 300 mM/l) and regulation is closely correlated with the osmotic potential of the soil (McClanahan, 1967, 1972; Shoemaker *et al.*, 1969). Hillman (1980) confirmed that the closely related *Scaphiopus couchii* is exceptionately well adapted to xeric conditions, displaying the greatest dehydration tolerance of the various anurans tested. McClanahan (1967) found that blood pressure, during the initial stages of dehydration remains constant. This indicates that the water lost comes primarily from interstitial fluids, with deficits possibly replenished from the bladder urine. McClanahan (1972) confirmed by experiment that *S.couchii* had the ability to change its body water potential by urea accumulation.

The rate of oxygen consumption steadily declines in the first few weeks of hibernation, with dormant *S.hammondi* (syn. *multiplicatus*) utilising only 20% of oxygen respired by resting individuals (Seymour, 1973). The fat body weight of *S.multiplicatus* is approximately 3% of total body weight in September, following feeding. The energy reserves stored in the fat body account for approximately half the energetic requirements of the dormant toads (Seymour, 1973). Eggs develop during hibernation, in preparation for emergence and immediate spawning, so

females have more energetic demands over winter. Seymour (1973) proposed that in conjunction with low energy requirements, hibernation over two years may be possible if the host has a large fat body, resorbs its gametes and the mobilises other body tissues.

Dimmitt & Ruibal (1980a) discovered that spadefoots respond to low frequency sound as the primary emergence cue for favourable environmental conditions. However, if the soil temperature fell below 20°C emergence was inhibited. A number of secondary factors, that modify the toads response to the primary factors were listed: 'temperature, amount of rain, intensity of rain, time of day, amount of rain on preceding days, and possibly time of year and changes in soil moisture'. Following hibernation, with heavy rains, toads move directly to breeding ponds, rarely feeding en route (Ruibal *et al.*, 1969).

Dimmitt & Ruibal (1980b) documented a natural diet of *S.multiplicatus* in the San Simon valley, of which termites accounted for 72% of wet weight, beetles 22%, ants 4%, and crickets, spiders, solpugids, scorpions, mites the remaining 4%. All the termites ingested were winged alates, therefore the contribution of this group to the overall diet depends upon the emergence of the winged morph. The quality of prey items also influences the assimilation efficiency of spadefoot toads. *S.couchii* has an assimilation efficiency of 95% when fed *Tenebrio* larvae but only 69% when fed *Tenebrio* beetles, due to higher levels of indigestible chitin (Dimmitt & Ruibal, 1980b). By calculation, Dimmitt & Ruibal (1980b) estimated that to survive hibernation, on average *S.multiplicatus* males require 7 and females

8.8 feeding nights. Furthermore, the female has high energetic demands to produce ovaries which are typically 20% of body weight (Seymour, 1973). Dimmitt & Ruibal (1980b) cited Jones (1978), who demonstrated that *S.multiplicatus* utilise twice as much energy as the larger *S.couchii* during hibernation, particularly with reference to their osmotic balance. Although not explained, one would expect this to be a direct result of the larger surface area to volume ratio of *S.multiplicatus*.

Due to the ephemeral nature of the desert ponds, spadefoot toads must exploit the summer monsoons at the earliest opportunity for successful breeding. The temporary ponds begin to fill on the first night of heavy rain and the toads emerge from their burrows to congregate in the pools (Bragg, 1965; Dimmitt & Ruibal, 1980a; Forester, 1973; Ruibal *et al.*, 1969).

Forester (1973, 1975) showed that females respond positively to the calls of conspecific males. In the San Simon valley, Simovich (1985) cited Sattler (1978) who noted male *S.bombifrons* call from the edge of a pond, resulting in a ring of males around the periphery. Male *S.multiplicatus* were found to be active searchers for mates, calling from floating positions. Variation in call position has also been noted in other areas (Bragg, 1945; Brown, 1976; Lowe, 1954 and Wasserman, 1957), for example males of the disjunct population of *S.bombifrons* in Texas call from open areas within the pond (Sattler, 1985; Wiens & Titus, 1991).

The overcrowding of breeding sites may occur and this mass convergence leads to the 'scramble competition' described by Wells (1977). Males may become indiscriminate and will amplex almost any object they come into contact with (Blair, 1958; Bragg, 1965). Simovich (1985) observed that once amplexed, males are very rarely dislodged. Therefore, once an interspecific match is made, the probable result is mating and fertilization. Theoretically, the direction of introgression should be biased towards the genome of *S.bombifrons* (as the males position themselves along the periphery of the pond, Sattler, 1978). In practice, however, Simovich (1985) found that due to the relative scarcity of *S.bombifrons* in this region, and the non-preferential behaviour of *S.multiplicatus* males the majority of heterospecific matings involved the latter. Tinsley (1989, 1990) stated that female *S.couchii* breed only once a season, with males entering breeding congresses on up to three separate occasions.

As an adaptation to arid environments spadefoot toads develop very rapidly. For example, *S.bombifrons* takes just 20 hours to hatch at 30^{0}C (Justus *et al.*, 1977 cited by Duellman & Trueb, 1986) and may reach metamorphosis in 13 to 15 days (King, 1960; Voss, 1961). However, differences between species do occur, Zweiffel (1968, 1977) found that at constant temperature *S.bombifrons* tadpoles reached metamorphosis 1, 2 and 8.5 days faster than *S.multiplicatus* at 21, 26 and 32^{0}C respectively. This is possibly an adaptation to their preferred lower elevations with the associated higher temperatures. Simovich (1985) also documented different developmental rates between tadpoles of varying parentage, with hybrid crosses possessing a development rate similar to that of *S.bombifrons*.

Trade-offs between developmental rate and size at metamorphosis could possibly explain the differences observed. However, Simovich (1985) found that the longer developmental time did not afford any significant size advantage to *S.multiplicatus*.

Members of the genus *Scaphiopus* have been found to have low DNA content per cell (Goin, Goin & Bachmann, 1968).

Such an attribute will be a major factor in allowing anurans to colonise arid regions, where breeding typically occurs in ephemeral waters.

Transmission opportunities for parasites using aquatic, infective stages are limited to the few breeding nights a year. Parasites using intermediate hosts will have the chance to infect *Scaphiopus* during their short activity period. Transmission by direct penetration will be hampered by the arid conditions and, during hibernation, the static host population. It is apparent that the ecology of the spadefoot toads may have a major influence on the parasite fauna they harbour.

Few parasite species have been recorded from the genus *Scaphiopus* (Table 4.1), therefore, the first remit of this study was to document the parasites present in the study area. Furthermore, in Europe, hybrid mice and fish have been found to harbour overwhelming worm burdens when compared to pure host species sampled outside the hybrid zone (Dupont & Crivelli, 1988; Moulia *et al.*, 1991, 1993; Sage *et al.*, 1986). Alternatively, within a hybrid zone, a non-susceptible parental genome (and closely associated hybrids) may harbour significantly fewer parasites than the competing (susceptible) genome (Coustau *et al.*, 1991; LeBrun *et al.*,

1992). The hybrid zone between *S.bombifrons* and *S.multiplicatus* provided an opportunity to assess the influence of host genetic constitution on susceptibility to parasitic infection.

4.3 Materials and Methods.

As described fully in Chapter 2, a number of different sites were used in both years of field collection. Those animals captured at each location were maintained in marked tanks and electrophoretically typed. A sub-sample of the collections were dissected and all parasites recorded (full methodology described in Chapter 3). Dissections of this nature accounted for 12 *S.bombifrons*, 105 *S.multiplicatus* and 6 F_1 hybrids from 12 sites in 1991; 13 *S.bombifrons*, 17 *S.multiplicatus* and 1 hybrid (*S.multiplicatus* backcross) from 2 sites in 1992; 154 specimens in total.

Using a dissection microscope, all major organ systems were examined for possible parasitic infection, in particular, the eyes, nostril sinuses, eustachian tubes, mouth, male vocal sac, glottis, lungs, stomach, intestine, rectum, urinary bladder and kidneys. Furthermore, a thorough search for encysted parasites was made, with the epidermis gradually removed and all major muscle blocks teased apart. Although blood samples were taken from hosts to measure the packed cell volume (PCV), no smears were made to check for protozoan/filarial blood parasites. The parasites recovered were fixed in the following manner: Monogenea were fixed under 22x22mm coverslips with sufficient pressure to display the hamulus profile, Digenea and Cestoda were flattened under a second microscope slide, in a 10% formal saline solution. Nematodes were fixed in hot 70% alcohol.

On return to Q.M.W. (London), monogeneans, digeneans and cestodes were stained, dehydrated and mounted *in toto* as described in Chapter 3. Nematodes were temporarily mounted in glycerol for identification. All parasites were identified to genus and where possible to species. A number of toads, returned from fieldwork in 1990, 1991 and 1992 from the same study sites, were maintained at Q.M.W. for experimental infection with *N.Scaphiopodis* (described in Chapter 3). At dissection, infection by other parasites was noted, providing a further sample for analysis and have been used to provide information used in the discussion of this chapter.

In addition, whilst in the field, gravid proglottids of *Distoichometra bufonis*, seperated from the strobila, were recovered from the rectum of a single *S.multiplicatus* (which harboured 3 adult cestodes in the duodenum) during routine dissection. Between 3 and 5 proglottids were pipetted via a blunt-ended dropper directly into the stomach of 6 conspecific recipient toads taken from road collections, which were dissected at 8, 9, 13 and 19 days post-infection (p.i.).

4.4 Results.

The range of parasite species recovered from all 25 *S.bombifrons*, 122 *S.multiplicatus*, 6 F_1 hybrids and 1 *S.multiplicatus* backcross over the two sampling years is recorded in Table 4.2. The prevalence and mean intensity of all parasites by sample year are summarised in Table 4.3.

Measurements of host snout-vent length (SVL) and body weight (B.wt) of all spadefoots dissected, in both study years are summarised below.

1991		*S.multiplicatus* (n = 105)	Hybrids (n = 6)	*S.bombifrons* (n = 12)
SVL / mm	mean	46.8	49.9	51.8
	S.D.	3.1	3.0	3.6
	range	39.0 - 53.0	46.0 - 55.0	44.0 - 58.5
B.wt/g	mean	10.9	12.9	14.3
	S.D.	2.3	2.1	2.8
	range	6.6 - 16.9	11.3 - 17.4	8.7 - 19.8

1992		*S.multiplicatus* (n = 17)	Hybrid (n = 1)	*S.bombifrons* (n = 13)
SVL / mm	mean	45.9	43.0	48.3
	S.D.	2.4	-	3.0
	range	43.0 - 52.0	-	43.0 - 53.0
B.wt/g	mean	9.9	9.0	12.2
	S.D.	1.4	-	1.9
	range	7.6 - 13.9	-	9.9 - 15.8

Table 4.4 Host dimensions of *Scaphiopus spp.* collected in 1991 and 1992.

As described fully in Chapter 3, the monogenean, *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 was recovered from both *S.bombifrons* and *S.multiplicatus* but not from hybrid genotypes.

Three distinct categories have been defined for *N.scaphiopodis* infection: group 1 refers to recently invaded stages in the respiratory tract, these worms having been acquired in the short period of spawning prior to collection; group 2 refers to recently migrated worms in the urinary bladder, which may be successful invasions from spawning earlier in the year of capture or worms from the previous year, which arrested in the respiratory tract over winter and were stimulated to migrate in the year of sampling; the final category, group 3 refers to adult stages which have been harboured by the host for at least one year in the urinary bladder.

Prevalences of *N.scaphiopodis* ranged from 0-15.4% (1-7 worms/host) and 0-19.0% with a range of 1-4 worms/host for groups 2 and 3 respectively (Table 4.3). Recent invasions in the respiratory tract ranged in prevalence from 0-60.0% (1-32 worms/host). Only group 1 worms were recovered from hybrid genotypes.

As described in detail in Chapter 5, metacercariae of the digenean, *Clinostomum complanatum* Rudolphi, 1819 were found encysted in the mesenteries of both *S.bombifrons* and *S.multiplicatus* but not hybrid genotypes. Prevalences in pure hosts ranged from 5.9-15.4% (1-18 worms/host) with a limited distribution, only Millers Pond, Number 1 Pond and Black Dog Pond harbouring infected spadefoots. This provides two new host records for *C.complanatum* and a new locality record for this region of Arizona.

The nematotaeniid cestode, *Distoichometra bufonis* Dickey, 1921 was rare and was only recovered from 3 specimens of *S.multiplicatus* at two sites, Luthers Field (1/3) and Bridge on Route 80 (2/3), infection levels ranged from 1-4 worms/host. This cestode has not previously been reported from *S.multiplicatus*.

Nematodes of the genus *Aplectana* Railliet & Henry, 1916 were recovered from the lower alimentary tract of all host types sampled. These worms were identified as *Aplectana itzocamensis* Bravo-Hillis, 1943 with prevalences ranging from 11.8-33.3% (1-25 worms/host). Frequency distributions (Fig.4.1) indicate a positively skewed population of parasites within all members of the host population, with those infecting *S.multiplicatus* approaching an overdispersed distribution. This represents new host records (*S.bombifrons* and F_1 hybrids of *S.bombifrons* x *S.multiplicatus*) for *A.itzocamensis*.

Third-stage larvae of the genus *Physaloptera* Rudolphi, 1819 were found firmly attached to the gastric mucosa of pure *S.multiplicatus* genotypes only. A frequency plot of *Physaloptera* within the random sample of *S.multiplicatus* collected in 1991 exhibits a positively skewed distribution (Fig.4.2), with 1-17 worms/host at an overall prevalence of 15.2%. Once again, the recovery of *Physaloptera sp.* from *S.multiplicatus* presents a new host record.

The microfilariae found in the blood of *S.multiplicatus* were the larvae of unidentified filarial nematodes, possibly inhabiting the kidneys. The presence of this larval stage was only noted by chance, blood released into dissection dishes

was observed under the dissection microscope. Larval nematode cysts were rare, with only 3 infected *S.multiplicatus*, all collected in 1991 (Table 4.3). The cysts were found embedded between the mucosa and outer musculature of the stomach, with a maximum of 52 cysts/host recovered.

The endocommensal rectal protozoans, *Nyctotherus sp./Balantidium sp.* and *Opalina sp.* were common. Simply the presence of these parasites was documented, with no distinction between *Nyctotherus sp.* or *Balantidium sp.* at dissection. Over the two study years, at different localities, 0-100% of the hosts dissected harboured rectal protozoans (Table 4.3).

No significant correlations between host factors and infection with *D.bufonis*, *A.itzocamensis* and larval nematode cysts were found. Infection with *N.scaphiopodis* and *C.complantum* are discussed in detail in Chapters 3 and 5 respectively. The host factors considered were: SVL; B.wt; fat body and gonad weight, which may indicate parasite-induced pathology. However, a correlation between SVL and infection with *Physaloptera sp.* was noted (Fig. 4.3). Regression analysis for SVL produces a small R^2 value of 6.1, however, this is significant as $p = 0.016$. A similar, weaker correlation was also found for body weight and *Physaloptera sp.* infection ($R^2 = 4.4$, $F = 4.27$, $p = 0.042$).

Comparisons on the basis of host sex were made using the largest pure species sample, *S.multiplicatus* collected in 1991. Of the 105 individuals dissected 30.5% were females. Table 4.5 records the infection levels of three helminths (*D.bufonis*,

A.itzocamensis and *Physaloptera sp.*). As documented in Chapter 2, the male toads were slightly smaller and lighter than the females (mean SVL 46.5:47.6mm & B.wt 10.69:11.46g respectively). For both nematode species, infection levels are comparable and individual Mann-Whitney tests between the sexes revealed no significant differences in their distribution. However, the rare *D.bufonis* was found only in 3 male specimens. Although hosts were collected from a number of breeding sites and roads, for the purposes of this survey sample sizes are too small to compare statistically.

The experimental infections of 6 *S.multiplicatus* with proglottids of *D.bufonis* were unsuccessful. All recipient toads were orally infected with 5 proglottids, except the specimen dissected at 9 d p.i. which received 3. Dissections of individual toads at 8, 9, 13 d p.i. and a pair at 19 d p.i. produced no indication of cestode establishment. The sixth host died during the experimental period.

4.5 Discussion.

The nematotaeniid cestode, *Distoichometra bufonis*, has been reported from a number of hosts in the southern U.S.A. Dickey (1921) described this parasite from *Bufo lentiginosus* (syn.*terrestris*) in Georgia. Brandt (1936) found infected *B.woodhousii fowleri* and *Scaphiopus holbrooki* in North Carolina. Five hosts in Texas have been documented; *B.speciosus*, *B.cognatus* and *S.couchii* by Kuntz (1941), in addition to *B.debilis debilis* and *B.woodhousi woodhousii* by McAllister, Upton & Conn (1989). Furthermore, Tinsley (1990) recorded *D.bufonis* from *S.couchii* in sympatry with the toads examined in this study.

The life-cycle of this cestode has not been elucidated. A direct life-cycle has been proposed for the closely related nematotaeniid, *Cylindrotaenia americana* Jewell, 1913 and supported by Stumpf (1982). Hardin & Janovy (1988) attempted to apply the experimental protocol of Stumpf (1982) to *D.bufonis* without success. The limited experimental infections in this study also proved unsuccessful. Hardin & Janovy (1988) commented on the lack of experimental evidence to support the direct life-cycle proposed for these cestodes and further investigation is required.

Hardin & Janovy (1988) reported a stable population of *D.bufonis* infecting *Bufo woodhousii*, once recruitment of metamorphs was accounted for, fitted an overdispersed distribution, typical of parasitic species (Crofton, 1971). Infection of toads as small as 20mm SVL was recorded, thus if an intermediate host is utilised by *D.bufonis* it must be of small proportions. Brandt (1936) found higher infection levels of *D.bufonis* in *Bufo woodhousii* than *Scaphiopus holbrooki*, and suggested that the difference may be explained by food preference. However, whatever mode of transmission employed, it is clear from the work of Hardin & Janovy (1988) that in certain regions, the life-cycle is readily completed in the wild. *Scaphiopus spp.* hibernation may last for 10 months (Tinsley, 1990) and this will minimise the detrimental effects of cestode infection, as the parasite will also be starved for this period. The chronic deprivation of nutrients will mean the loss of virtually all proglottids, and although the strobila may persist, new proglottids will have to be produced during the short period of host feeding, further delaying opportunities for transmission. This may be a major influence in producing the low infection levels observed.

Aplectana itzocamensis was the most common nematode recovered in this study. This species was first described from *S.multiplicatus* by Bravo-Hillis (1943) from Puebla, Mexico and has been reported from one other spadefoot toad, *S.couchii* in the San Simon valley (Tinsley, 1990) which is sympatric and syntopic with the toads examined in this study. Baker (1985) redescribed *A.itzocamensis* from an alternative host, *Bufo woodhousii*, and there have been four other bufonid hosts recorded: *B.alvarius*, *B.cognatus* and *B.punctatus* in southern Arizona (Goldberg & Bursey, 1991a, 1991b) and *B.marinus* in Costa Rica (Brenes & Bravo-Hillis, 1959).

The life-cycle of *A.itzocamensis* has not been characterised. Goldberg & Bursey (1991b) cited the work of Chabaud & Brygoo (1956) on *A.courdurieri*. For this species a two stage life-cycle was proposed, the first stage is free-living, encompassing the hatching stage to the third larval moult. The parasitic stage, incorporates the infective third-stage to the parasitic adult, the latter inhabiting the rectal lumen of the host. The mode of infection is thought to be by ingestion, either during feeding as a tadpole or by the accidental swallowing of infective stages by adult toads. The habitat of this nematode indicates that mainly rectal contents are ingested and there are no records of host tissue being ingested, therefore there should be no competition with the host for resources.

The mode of transmission of *A.itzocamensis* is unclear. Spadefoot toads collected in the field, and maintained in the laboratory, do not provide any distinct indicators as to the method(s) of transmission. The vast majority of toads kept at

Q.M.W. were exposed to swimming oncomiracidia of *N.scaphiopodis* (Chapter 3) and as a result they may have ingested or been actively penetrated by infective nematode larvae during aquatic experimental procedures. However, it is clear from lab. records that infection may span at least two years, demonstrated by toads maintained and fed individually (on U.K. bred crickets). Juvenile toads collected from the field, maintained individually and dissected a year later may be infected. This suggests that tadpoles may acquire *A.itzocamensis* prior to metamorphosis or if the infective stage uses an intermediate host, it must be of a size to be consumed by the smallest toads. The possibility of active penetration and autoinfection remains and further experimental studies are required.

Physaloptera sp. although recovered only from *S.multiplicatus* in the field, has been found in *S.bombifrons* during routine laboratory dissections. This nematode has also been reported from *S.couchii* in the same locality as this study (Tinsley, 1990). Baker (1989) suggested that physalopterids are often recovered from paratenic hosts, which is the most probable explanation for those worms recovered from *S.multiplicatus*. The life-cycle of physalopterids involves an intermediate host, for example, crickets or cockroaches (Schmidt & Roberts, 1989), which may become prey items for *Scaphiopus* as well as the correct (definitive) host. The ingestion of prey items may explain the significant relationship ($p = 0.016$) between SVL and physalopterid infection. If SVL is taken as an indication of host age, then older toads potentially have a greater exposure to infection, by the number of prey items eaten. The skeletochronological aging of the field sample of *S.multiplicatus* may provide a more accurate age structure of the host population,

possibly clarifying the trend presented here. The positively skewed distribution of *Physaloptera sp.* (Fig.4.2) may be the result of food preference or by the chance encounter of infected prey.

Schorr, Altig & Diehl (1990) found that anuran tadpoles acquire opalinid infection soon after the differentiation of the mouthparts, however, infection with *Nyctotherus cordiformis* was more variable. Rapidly developing tadpoles (typical of *Scaphiopus spp.*) show a strong positive population increase over time. No correlation between *N.cordiformis* and the opalinid was found, leading the authors to conclude that they 'respond differently in the same environment'. In this study the prevalence of opalinid infection was much lower than that *Nyctotherus sp./Balantidium sp.*

Brandt (1936) and Campbell (1968) suggested that the diversity of parasitic species found in amphibians was directly related to their ecology, with greater diversity in the most aquatic species. However, Campbell (1968) found that terrestrial species harboured heavier burdens than aquatic species. The spadefoot toads of all genotypes were of comparable size in both study years, but for accurate examination of the age structure of the populations skeletochronology may prove a more accurate tool. The absence of significant differences on the basis of host genotype and sex for nematode and cestode species may be explained by host ecology. Spadefoot toads are voracious feeders, even ingesting noxious solpugid spiders and centipedes (Dimmitt & Ruibal, 1980b). In the brief period available to replenish their reserves, spadefoots must be almost indiscriminate about prey

selection. Both species share the same habitat and so will be exposed to the same range of parasites. The exceptions were either rare (*D.bufonis* & nematode cysts), or not present in the random samples taken, subsequently recovered in lab. animals (*Physaloptera sp.*). In desert ecosystems with almost exclusively terrestrial hosts, parasites have an extremely limited opportunity for transmission, leading to the restricted fauna recorded in this study.

Hybrid susceptibility to infection, has been documented by Sage *et al.* (1986) and Moulia *et al.* (1991, 1993) for mice, and Dupont & Crivelli (1988) for fish. In addition, LeBrun *et al.* (1992) and Coustau *et al.* (1991) working in regions of hybridisation, have reported that a particular parental genome (and their closely related hybrids) may be resistant to infection by particular parasites.
Hybrid susceptibility cannot be assessed in this study, due to the limited number of hybrid genotypes recovered. Of the 7 hybrids examined, only the helminths *A.itzocamensis* and juvenile *N.scaphiopodis* were recovered, both infection levels comparable to pure host genotypes (Tables 4.2, 4.3). Furthermore, there was no clear indication of pure species exhibiting differential infection levels for any of the parasite species recovered. Therefore there is no indication, from the limited data presented, that there are changes in genetic predisposition to infection which are associated with the *S.bombifrons/S.multiplicatus* hybrid zone.

This study has more than doubled the number of recorded parasites of *S.bombifrons* and added two species to the list for *S.multiplicatus*. However, it should be reiterated that apart from *N.scaphiopodis*, the remaining parasite species are either broad spectrum, non-specific generalists (*D.bufonis*; *A.itzocamensis*; *Nyctotherus sp./Balantidium sp.* and *Opalina sp.*) or utilise spadefoot toads as intermediate hosts (*C.complanatum*; possibly microfilarial nematodes) or paratenic hosts (*Physaloptera sp.*; possibly larval cysts).

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Scaphiopus bombifrons	Site of Infection.
Monogenea.	
Neodiplorchis scaphiopodis (Rodgers, 1941) Yamaguti, 1963	urinary bladder
Protozoa.	
Protoopalina scaphiopodos[1]	rectum
Scaphiopus multiplicatus	
Monogenea.	
Neodiplorchis scaphiopodis (Rodgers, 1941) Yamaguti, 1963	urinary bladder
Cestoda.	
Cylindrotaenia americana Jewell, 1916	intestine
Nematoda.	
Aplectana hoffmanni Bravo-Hillis, 1943	intestine
Aplectana itzocamensis Bravo-Hillis, 1943	intestine
Protozoa.	
Opalina oblanceolata Metcalf, 1923	rectum
Protoopalina hammondi[1]	rectum
Trichomonas augusta Alexeieff, 1911	rectum
Scaphiopus couchii	
Monogenea.	
Pseudodiplorchis americanus Rodgers & Kuntz, 1940	urinary bladder
Cestoda.	
Distoichometra bufonis Dickey, 1921	intestine
Nematoda.	
Aplectana incerta Caballero, 1949	intestine
Aplectana itzocamensis Bravo-Hillis, 1943	intestine
Oswaldocruzia pipiens Walton, 1929	intestine
Physaloptera sp. Rudolphi, 1819	stomach
Scaphiopus holbrooki	
Cestoda.	
Distochometra bufonis Dickey, 1921	intestine
Protocephalid cysts	intestinal wall
Nematoda.	
Agamonena sp.	general
Cosmocercoides dukae Holl, 1928	intestine
Oswaldocruzia leidyi Travassos, 1917	intestine
Oswaldocruzia pipiens Walton, 1929	intestine
Physaloptera sp. Rudolphi, 1819	stomach
Rhabdias sp. (larval)	body cavity
Rhabdias ranae Walton, 1929	lungs
Protozoa.	
Nyctotherus cordiformis Ehrenberg, 1838	intestine
Octomitus intestinalis Prowazek, 1904	intestine
Opalina carolinensis Metcalf, 1923	intestine
O.oblanceolata Metcalf, 1923	intestine
O.obtrigonoidea Metcalf, 1923	intestine
O.triangulata Metcalf, 1923	intestine
Trichomonas augusta Alexeieff, 1911	intestine

Table 4.1. The parasitic fauna of the spadefoot toads, *Scaphiopus spp.* Complied from Brandt, 1936; Brooks, 1976; Golberg & Bursey, 1991a; Kuntz, 1941; Tinsley, 1990 & Walton, 1964. Note: [1] = authority not stated.

	S.multiplicatus (n = 122)	Hybrids (n = 7)	*S.bombifrons* (n = 25)
Monogenea	*Neodiplorchis scaphiopodis*		*Neodiplorchis scaphiopodis*
Digenea	*Clinostomum complantum*		*Clinostomum complantum*
Cestoda	*Distoichometra bufonis*		
Nematoda	*Aplectana itzocamensis*	*Aplectana itzocamensis*	*Aplectana itzocamensis*
	Physaloptera sp.		
	Unidentified filarial nematodes		
	Larval cysts		
Protozoa	*Balantidium sp./ Nyctotherus sp.*	*Balantidium sp./ Nyctotherus sp.*	*Balantidium sp./ Nyctotherus sp.*
	Opalina sp.	*Opalina sp.*	*Opalina sp.*

Table 4.2 The parasite fauna of *Scaphiopus bombifrons*, *S.multiplicatus* and their hybrids (pooled sample of 6 F_1 and 1 backcross *S.multiplicatus*) recovered from field collections in 1991 and 1992 in the San Simon valley, Arizona/New Mexico, U.S.A.

A.

	S.multiplicatus (n =105)		Hybrids (n = 6)		*S.bombifrons* (n = 12)	
	prev.	int.	prev.	int.	prev.	int.
N.scaphiopodis Gr 1	60.0	5.7	50.0	4.7	50.0	7.7
Gr 2	2.9	1.3	0	-	8.3	1.0
Gr 3	19.0	1.7	0	-	16.7	1.0
C.complanatum	9.5	10.5	0	-	8.3	1.0
D.bufonis	2.9	2.7	0	-	0	-
A.itzocamensis	27.6	4.1	33.3	5.5	16.7	13.0
Physaloptera sp.	15.2	4.3	0	-	0	-
Larval cysts	2.9	29.3	0	-	0	-
Balantidium sp. /Nyctotherus sp.	53.3	-	100.0	-	75.0	-
Opalina sp.	26.7	-	16.7	-	25.0	-

B.

	S.multiplicatus (n =17)		Hybrids (n = 1)		*S.bombifrons* (n = 13)	
	prev.	int.	prev.	int.	prev.	int.
N.scaphiopodis Gr 1	58.8	2.5	0	-	23.1	4.3
Gr 2	11.8	1.0	0	-	15.4	4.5
Gr 3	0	-	0	-	7.7	1.0
C.complanatum	5.9	14.0	0	-	15.4	11.5
D.bufonis	0	-	0	-	0	-
A.itzocamensis	11.8	1.5	0	-	0	-
Physaloptera sp.	0	-	0	-	0	-
Larval cysts	0	-	0	-	0	-
Balantidium sp. /Nyctotherus sp.	35.3	-	0	-	46.2	-
Opalina sp.	23.5	-	0	-	7.7	-

Table 4.3 Infection levels of the parasitic fauna of *S.bombifrons*, *S.multiplicatus* and their hybrids collected in the San Simon valley, Arizona/New Mexico, U.S.A in A.) July-August, 1991 and B.) July, 1992. (Abbreviations: prev. = prevalence expressed as a percentage; int. = mean intensity; Gr 1, Gr 2 & Gr 3 = groups 1, 2 & 3).

		Male (n = 73)	Female (n = 32)
D.bufonis	prev.	4.1	0
	int.	2.7	-
A.itzocamensis	prev.	28.8	18.8
	int.	4.3	4.2
Physaloptera sp.	prev.	15.1	15.6
	int.	3.8	5.4

Table 4.5 Infection levels of *D.bufonis*, *A.itzocamensis* and *Physaloptera sp.* for male and female *S.multiplicatus* collected in July-August, 1991. (Abbreviations: prev. = prevalence expressed as a percentage; int. = mean intensity).

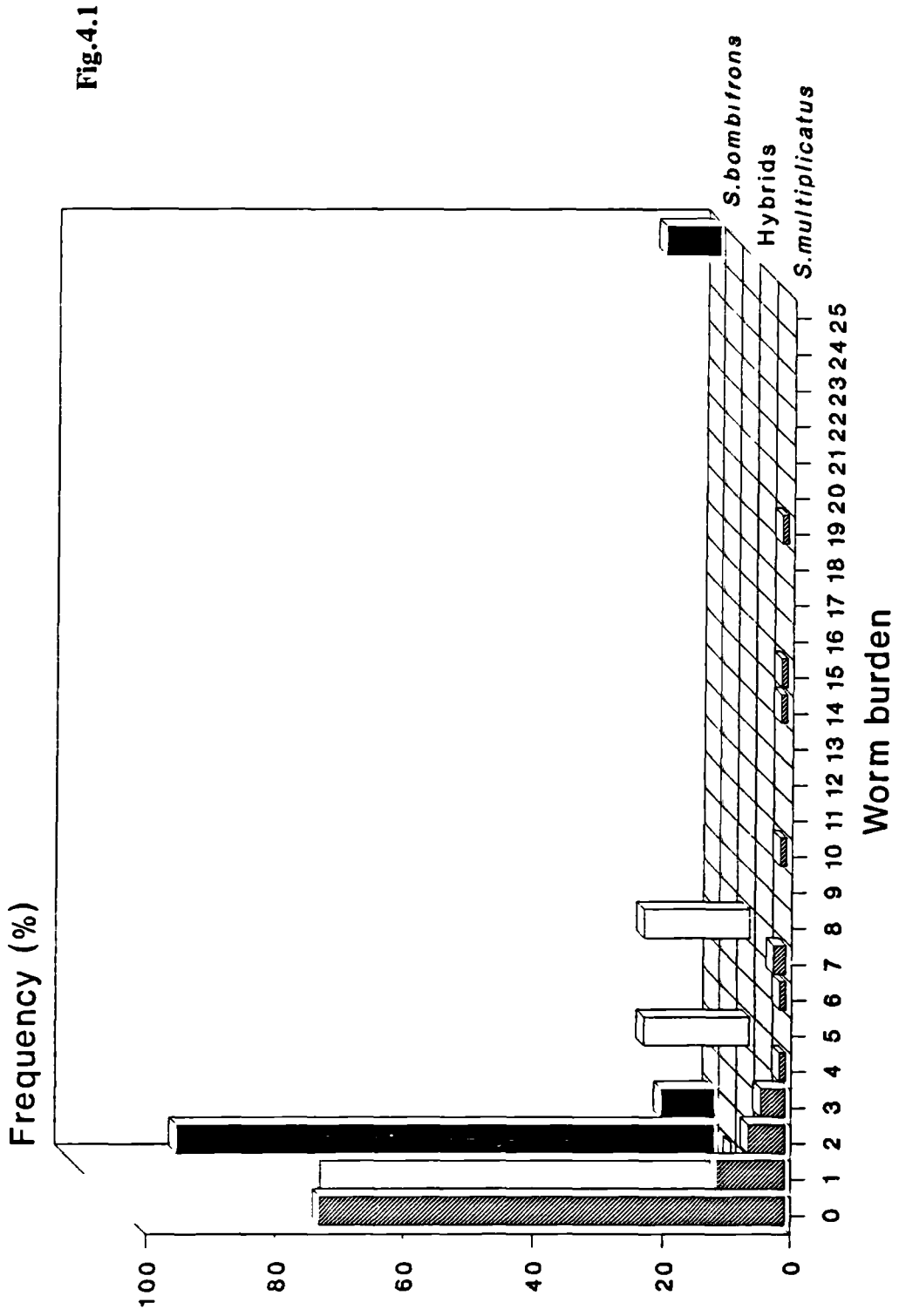
4.7 Legends.

Fig.4.1 Frequency distribution of *Aplectana itzocamensis* in the spadefoot toads, *S.bombifrons* (n = 12), *S.multiplicatus* (n = 105) and their hybrids (n = 6) collected in 1991 from the San Simon valley, Arizona/New Mexico, U.S.A.

Fig.4.2 Frequency distribution of larval *Physaloptera sp.* in the spadefoot toad, *S.multiplicatus* (n = 105) collected in 1991 from the San Simon valley, Arizona/New Mexico, U.S.A.

Fig.4.3 Relationship between snout-vent length (SVL) of *S.multiplicatus* (n = 105) and infection with larval *Physaloptera sp.* collected in 1991 from the San Simon valley, Arizona/New Mexico, U.S.A. (R^2 = 6.1%, F = 6.02, p =0.016).

Fig.4.1


Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
Worm burden
S.bombifrons
Hybrids
S.multiplicatus

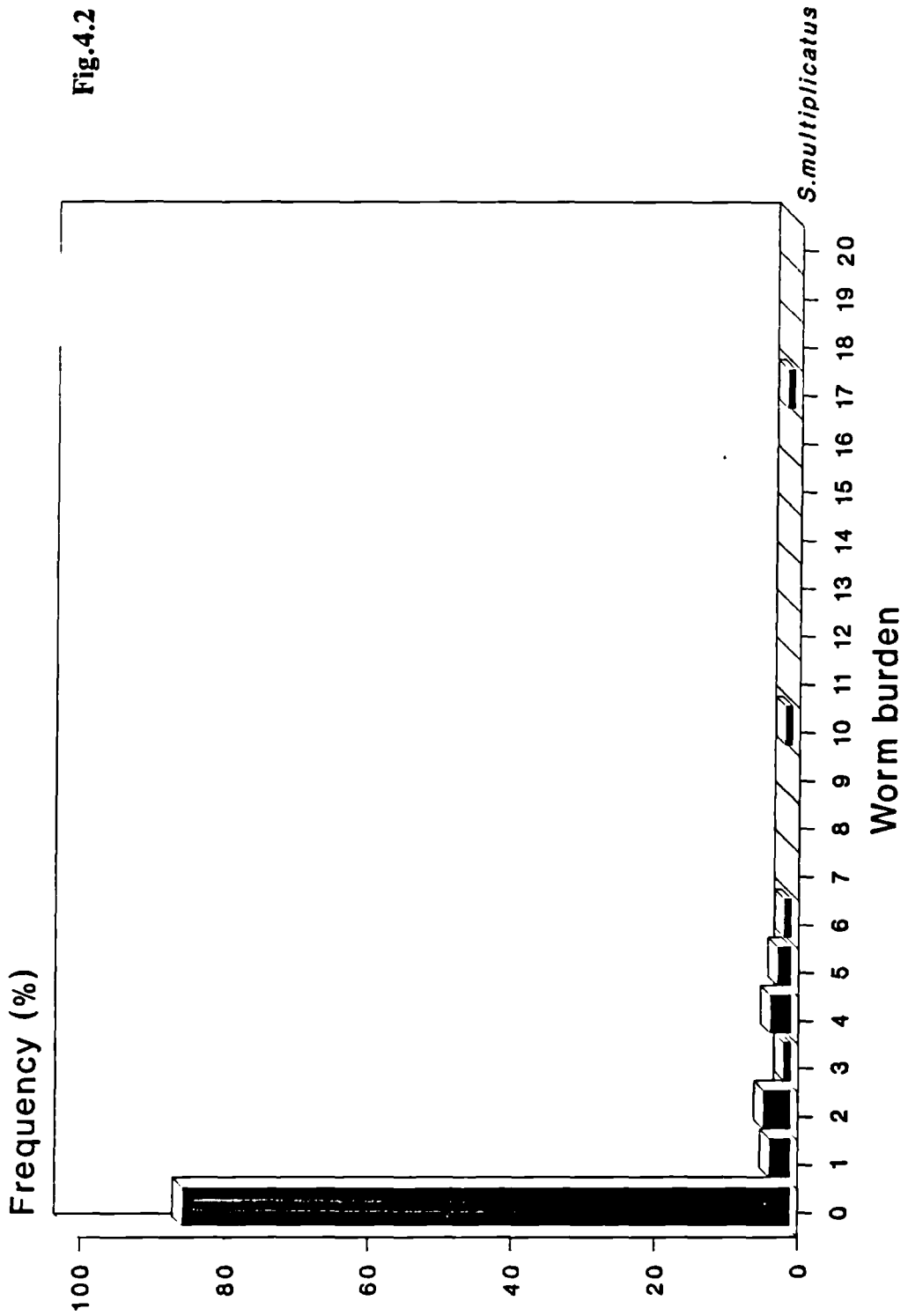
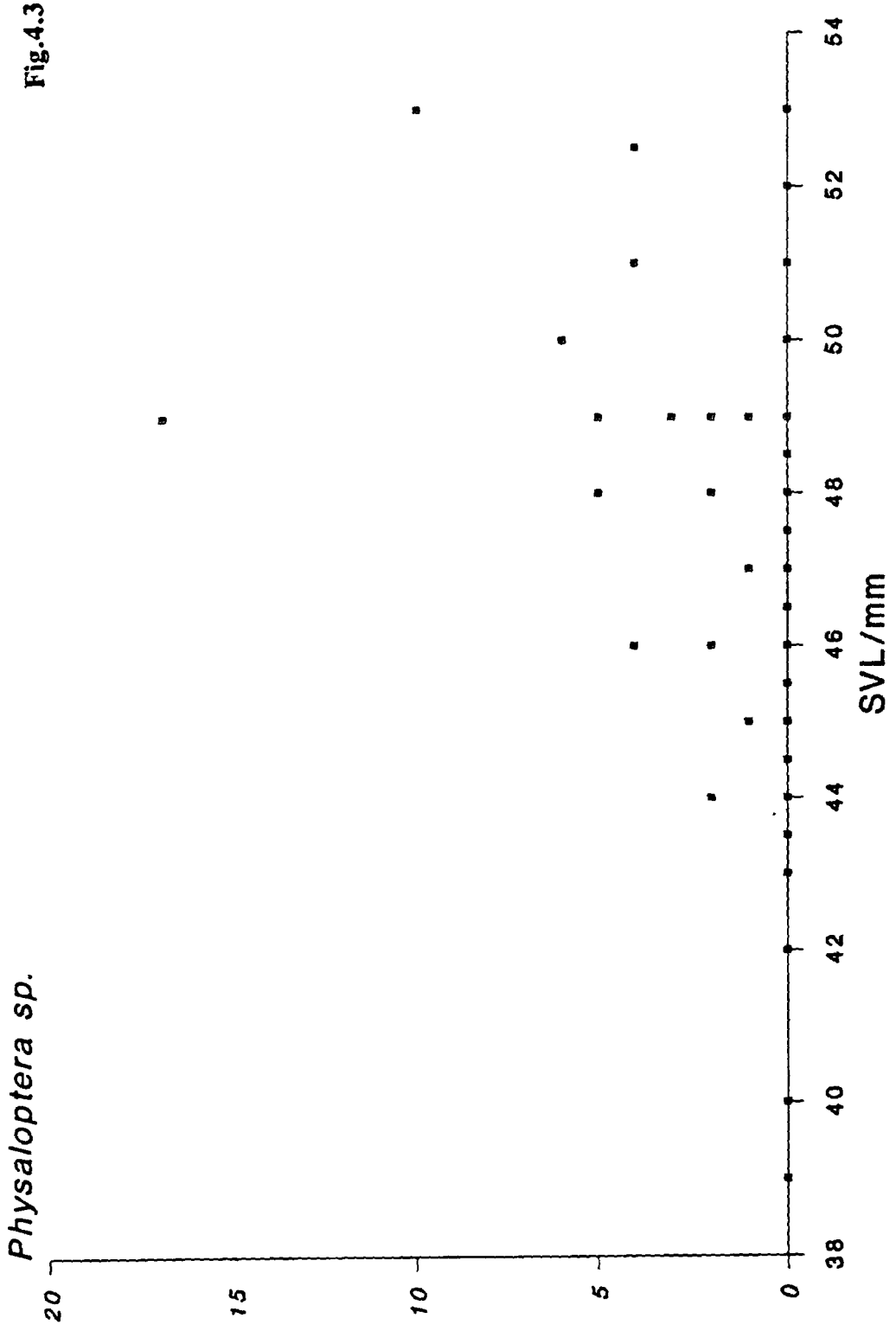

Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
Worm burden
S.multiplicatus

Fig.4.2

Fig.4.3



Chapter 5.

The introduction of the digenean *Clinostomum complanatum* (Rudolphi, 1814) (Trematoda:Digenea) into a desert environment.

5.1 Abstract.

Digeneans are typically absent from desert ecosystems because of the unfavourable conditions for molluscan intermediate hosts. A complex ecotone of desert biotas is found in the San Simon Valley in southeastern Arizona, U.S.A. and the low annual rainfall permits no natural permanent waterbodies. Cattle ranching in the area has resulted in a number of habitat changes, particularly the pumping of bore water into permanent ponds for livestock. Previously, the ephemeral nature of water bodies in the valley had restricted the amphibian fauna to desert-adapted species, such as the spadefoot toads, *Scaphiopus bombifrons*, *S.couchii* and *S.multiplicatus*. However, the permanent ponds have allowed aquatic snails and an introduced amphibian, the bullfrog *Rana catesbeiana* to become established.

This study documents the recovery of digenean metacercariae of *Clinostomum complanatum* Rudolphi, 1814 from *R.catesbeiana*, *S.bombifrons* and *S.multiplicatus*. At individual sites, infection levels of *C.complanatum* in *R.catesbeiana* may reach a prevalence of 100% with intensities of 1-114 worms/host. For pooled samples of *S.bombifrons* and *S.multiplicatus* prevalence may reach 27.7% with burdens of 1-18 worms/host. This is the first record of digenean infection of *Scaphiopus spp.* in the area and has implications for endemic amphibians.

5.2 Introduction.

A complex ecotone of desert biotas is found in the San Simon Valley (altitude 1280-1520m) in southeastern Arizona, U.S.A (Dimmitt & Ruibal, 1980). This region receives an annual rainfall of approximately 220mm, most of which is concentrated into a few weeks following months of drought, with characteristically sudden and torrential falls (Ruibal, Tevis & Roig, 1969).

The ephemeral nature of the water bodies formed by the monsoons restricts the amphibian fauna to desert-adapted species. There are six naturally occurring amphibian species in the San Simon valley, *Scaphiopus bombifrons*, *S.couchii*, *S.multiplicatus*, *Bufo alvarius*, *B.cognatus* and *B.debilis*. In order to survive the xeric conditions the spadefoot toads, *Scaphiopus spp.*, typically hibernate underground for 8 to 10 months of the year, only emerging after heavy rain to feed and reproduce (Dimmitt & Ruibal, 1980). On breeding nights, toads only enter water after darkness and leave before dawn, a maximum of 7 hours in this region. Females only breed once a season and males mate on up to 3 occasions a year. Therefore, host behaviour restricts the transmission opportunities for aquatic infective stages to less than 24 hours per annum (Tinsley, 1989).

The transient nature of standing water in desert regions prevents the successful establishment of molluscs. As a direct result digeneans, which utilise molluscs in their life-cycle, are also unable to persist. However, cattle ranching in the valley has resulted in a number of habitat changes, particularly the collection of water into permanent ponds for livestock (Simovich, 1985). As a direct result, molluscs

and the amphibian *Rana catesbeiana*, which could not otherwise survive the arid conditions, have been introduced and successfully established.

This study records trematodes of the genus *Clinostomum* Leidy (1856) in members of the autochthonous anuran fauna, *S.bombifrons* and *S.multiplicatus* as well as the allochthonous *R.catesbeiana*. Two species of *Clinostomum* have been reported from the United States: *C.attenuatum* Cort, 1913 and *C.marginatum* Rudolphi, 1819 (see McAllister, 1990). The latter is a junior synonym of *C.complanatum* Rudolphi, 1814 (Baer, 1933; Dowsett & Lubinsky, 1980; Feizullaev & Mirzoeva, 1983; Manter, 1938; Ukoli, 1966; Yamaguti, 1933), but is still widely used in the U.S.A. McAllister (1990) concluded that *C.marginatum* 'may persist as a viable subjective synonym'.

In North America, the first intermediate hosts of the Clinostomatidae are snails of the genus *Helisoma* and *Radix* (Hunter & Hunter, 1934; Yamaguti, 1971). Amphibians or fishes may be utilised as the second intermediate host depending upon the species of parasite (Cort, 1913), the definitive host commonly being a piscivorous bird. The number of species in the genus *Clinostomum* is a matter of conjecture, Feizullaev & Mirzoeva (1983) suggested that the numerous species described '..were identified by unacceptable characters and are all representatives of the cosmopolitan species *C.complanatum* which may develop in fish and reptiles'. However, other authors have proposed that a number of distinct, specific species do occur (Baer, 1933; Cort, 1913; McAllister, 1990; Ukoli, 1966). In addition to documenting clinostomatid infection in the San Simon valley, this study

includes a morphological description of the metacercariae and this is related to published accounts of *C.attenuatum* and *C.complanatum* /*C.marginatum*.

5.3 Materials and Methods.

Fieldwork was based at the Southwestern Research Station (S.W.R.S.), Portal, Arizona from 1st July to 17th August 1991 and 26th June to 28th July 1992. In 1991, a total of 243 spadefoot toads (*S.bombifrons*, *S.multiplicatus* and their hybrids) were collected from 14 study sites in the nearby San Simon valley. The sites are fully detailed in Chapter 2, this study utilised the following sites: Animas Road (n = 1); Black Dog Pond (n = 19); Bridge on Route 80 (n = 5); Dancers Tank (n = 33); Luthers Field (n = 4); Millers Pond (n = 65); Next to Windmill Pond (n = 12); Number 1 Pond (n = 16); Painted Mountain Road (n = 2); Peach Orchard Road (n = 11); Portal Road (n = 37); South of Rodeo (n = 14); State Line Road (n = 8) and Sulphur Draw (n = 16). Of the 141 dissected in the laboratory at S.W.R.S., 123 were dissected for the parasitological survey documented in Chapter 4 and the remaining 18 were used for experimental infections of *N.scaphiopodis*, *P.americanus* and *D.bufonis*. A further 102 were examined for digenean infection at Q.M.W., London.

In addition, 17 *R.catesbeiana* were collected from 2 sites and dissected at S.W.R.S. The collection sites are mapped in Fig.5.3 and summarised below, with sample size per site in brackets.

A. Willow Pond, cattle tank, 0.05km N of unpaved road at Sulphur Draw, 0.2km jct of State Line Road, (n = 15).

B. Windmill Pond, cattle tank, 0.2km NE of Portal Road, 1km NW jct of State Line Road, (n = 2).

In 1991, in an attempt to identify the first intermediate host(s) of *Clinostomum* in this region, snails were collected from Willow Pond and Windmill Pond and maintained in the lab. in beakers filled with pond water. A cold-light source was positioned overhead (to provide extra daytime illumination at an equivalent photoperiod) and the water examined microscopically for cercarial emergence.

In 1992, 55 *S.bombifrons*, *S.multiplicatus* and their hybrids were collected from 3 sites, Millers Pond (n = 20); Skeleton Canyon Road (n = 22); Sulphur Draw (n = 13); 31 were dissected in the laboratory at S.W.R.S. and a further 22 at Q.M.W. Five *R.catesbeiana* were collected from Willow Pond and dissected at S.W.R.S.

In both years the majority of specimens (214/298) were collected following heavy rain from breeding ponds, the remaining animals were gathered from the roads. There was no possibility of further transmission after capture. At dissection the anurans were examined for helminths, with the sites and number of cysts containing digenean metacercariae recorded (dissection procedure as described in Chapter 3). These robust worms were mechanically excysted with the aid of a seeker and fixed in 10% formal saline solution, under a glass slide weighted with a solid watchglass. At Q.M.W. worms were stained, dehydrated and mounted *in toto* as described in Chapter 3.

5.4 Results.

Encysted metacercariae were found in all regions of the body: eye sockets, glottis, mouth, jaw articulation, internal organs, subcutaneous on internal and external body musculature and from the limbs. The cysts were opaque, fibrous, often covered with pigment spots and were small in relation to the enclosed metacercariae. Worms removed from formalin-fixed cysts were folded, typically with both the anterior and posterior extremities bent back over the body.

A representative metacercaria recovered from *S.multiplicatus* is illustrated in Fig.5.1. Upon release from the cyst, worms were able to extend their bodies approximately twice their contracted length, with a maximum of 13mm recorded. Both the fore and hind-body were concave when viewed dorsally, this was particularly pronounced when the worm was extended. The oral sucker in contracted specimens rests in a distinctive muscular collar (Figs.5.1 & 5.2). The excretory system was distinguished by the complex network of fine creamy tubules which ramified through the body. The body movements of the metacercariae resulted in this material being expelled from the excretory pore. The definition of this system was lost in stained specimens. The reproductive system, although immature, was well defined in all stained specimens (Fig.5.1).

All 501 metacercariae recovered from *R.catesbeiana* were alive and active following excystment. Of the 147 cysts recovered from *Scaphiopus spp.* (*S.bombifrons* and *S.multiplicatus*) dissected at S.W.R.S., most were active, however, one host harboured 9 cysts containing dead metacercariae.

The worms from both *R.catesbeiana* and *Scaphiopus spp.* were identified as *Clinostomum complanatum* (Rudolphi, 1819) based on descriptions by Osborn (1912), Cort (1913), Ukoli (1966), Kagei *et al.* (1984) and Feizullaev & Mirzoeva (1983). Table 5.1 summarises the dimensions of representative sample of 40 *C.complanatum* metacercariae (20 taken from *R.catesbeiana* and 20 from *S.multiplicatus*). The overall body shape of live and fixed specimens was broad (Fig.5.1), and fine spines covered the cuticle. No cercariae were released from the snails maintained in the lab., thus the identity of the molluscan intermediate host(s) in this ecosystem remains unresolved.

A survey of ponds in this region of the San Simon valley revealed digenean infection at 5/16 locations. Infection levels by host species, host group (*Scaphiopus spp.*), site and sampling year are summarised in Table 5.2.

Willow Pond (pond A, Fig.5.3) is the only permanent waterbody in the vicinity and harbours a large population of *R.catesbeiana*. The size of frogs caught in both years varied considerably, mean SVL = 103.5mm (S.D. = 49.0) in 1991 and mean SVL = 55.6mm (S.D. = 12.3) in 1992. The prevalence of *C.complanatum* was 93.3% (1-114 worms/host) in 1991 and 100% (4-13 worms/host) in 1992 (Table 5.2 A). Infected spadefoot toads were not found in the immediate environs of this pond. Windmill Pond (pond B) is a large but ephemeral waterbody containing a small population of *R.catesbeiana*, only two frogs were caught from this site in 1991 but both were infected with *C.complanatum* (7 and 56 worms/host). 12 spadefoot toads were collected within a few metres of Windmill

Pond, all were uninfected (Fig.5.3). Apart from ponds A and B, the presence of bullfrogs was noted close to Millers Pond (on Portal road and State Line road) and at Black Dog Pond.

Ponds 8, 10 and 14 (Number 1 Pond; Millers Pond and Black Dog Pond respectively) are the only ponds where infected *Scaphiopus spp.* were recovered (Fig.5.3). In 1991 at Black Dog Pond and Number 1 Pond, a single infected *S.multiplicatus* was found at each site, with burdens of 4 & 3 worms/host respectively (Table 5.3B). The larger samples taken at Millers Pond exhibited infection levels of 1-18 worms/host at a prevalence of 27.7% (Table 5.2C) and was the only locality in which infected *S.bombifrons* were recovered. In 1992, only Millers Pond harboured toads infected with *C.complanatum*, at a prevalence of 25.0% and burdens of 2-15 worms/host. The variation in host SVL and body weight for *Scaphiopus spp.* was much less pronounced than for *R.catesbeiana* (refer to Table 4.2, Chapter 4). By regression analysis, no correlation was found between the intensity of infection by *C.complanatum* and the snout-vent length (SVL) or body weight (B.wt.) of *R.catesbeiana* (Table 5.3).

1991)	Host SVL v infection: R^2 = 6.5%, F = 1.05, p = 0.323 ,n = 17.
1991)	Host B.wt. v infection: R^2 = 8.7%, F = 1.42, p = 0.252, n = 17.
1991+1992)	Host SVL v infection: R^2 = 1.3%, F = 0.26, p = 0.616, n = 22.
1991+1992)	Host B.wt. v infection: R^2 = 3.2%, F = 0.67, p = 0.424, n = 22.

Table 5.3 Regression analysis of host dimensions and infection by *C.complanatum.*

With the uniformity of host dimensions and restricted possibility for transmission, it was not deemed appropriate to apply this analysis to *Scaphiopus spp.*.

For samples taken in 1991, frequency distributions of *C.complanatum* were positively skewed for *R.catesbeiana* (Fig.5.4A) and approached a poisson distribution for *Scaphiopus spp.* (Fig.5.4B). With regard to sites where infected spadefoot toads were recovered, pure parental and hybrid genotypes of *Scaphiopus spp.* were present at ponds 8, 10 & 14, although no hybrids were recovered from Millers Pond in 1992. Sample size varied between sites and sample years but no infected hybrid genotypes have been recorded. Sampling in two consecutive years at Millers Pond showed equivalent levels of infection (Table 5.2C), however, the constituent samples exhibited wide variation between the two collections (Table 5.2B), which will be discussed in relation to sample size. Furthermore, although a new sampling site (Skeleton Canyon Road, see Chapter 2) was sampled in 1992, over 36% of all *Scaphiopus spp.* collected were from Millers Pond, contributing to the similar infection levels of *C.complanatum* at all sites between the two years of sampling (Table 5.2C).

5.5 Discussion.

As noted in the Introduction, taxonomic reviews (Baer, 1933; Dowsett & Lubinsky, 1980; Feizullaev & Mirzoeva, 1983; Ukoli, 1966; Yamaguti, 1933) have suggested synonymy between *C.marginatum* and *C.complanatum*. Feizullaev & Mirzoeva (1983) proposed that *C.complanatum* encompassed both *C.attenuatum* and *C.marginatum* as synonyms. However, McAllister (1990) recognised two species in the U.S.A.: *C.attenuatum* and *C.marginatum* (whilst supporting the synonymy of *C.marginatum* and *C.complanatum*).

Osborn (1912) found little variation between metacercariae recovered from frogs and fish, regarding all to be examples of *C.marginatum*. However, Cort (1913) described a new species from frogs, *C.attenuatum* and listed 12 features which separated this from *C.marginatum*. These included host specificity, the position of the cyst in relation to host musculature, overall body shape, ratios of oral and ventral sucker diameters, body length and width ratios, position of the anterior tip of the uterus, tegmental spine morphology. Ukoli (1966) indicated that the most important of these features were host specificity, body shape and tegmental spine morphology. Larsen & Uglem (1988) also suggested that there were physiological differences between *Clinostomum* recovered from fish and frogs, an assumption based on comparisons of their data with those of Fried & Foley (1970).

In contradiction to the strict host specificity proposed for *C.marginatum* by Cort (1913) and Ukoli (1966), in North America second intermediate hosts of *C.marginatum* have been reported from a number of groups other than fish. These

include ranid frogs (Fortner, 1923; Fried & Foley, 1970; Manter, 1938; Osborn, 1912), hylid frogs (Hausfater, Gerhardt & Klump, 1990), salamanders (Fowler, 1947; McAllister, 1990), a three-toed amphiuma (Bennett & Humes, 1938), a plains garter snake (Hopkins, 1933), a siren (Manter, 1938).

Regarding overall body shape, the worms recovered in this study exhibited a variety of forms, depending upon their state of extension at the time of death, although no thin, uniform specimens, such as those illustrated by Cort (1913) for *C.attenuatum* were found. The metacercariae in this study are larger than those recorded in the literature, however, this is most probably due to the method of fixation. Morphological ratios contribute to the basis of specific identity (Cort, 1913) and they are unaffected by fixation, all fell within the range described for *C.marginatum*. Another significant diagnostic feature proposed by Cort (1913) and reiterated by Ukoli (1966) concerns tegmental spine morphology. Metacercariae recovered from piscine hosts possess fine spines and those from amphibians have coarse spines. However, fine and coarse spines have been recorded from metacercariae infesting frogs (Larsen & Uglem, 1990). The spines in this study were fine and their overall length falls within the range of *C.marginatum* proposed by Cort (1913).

The worms recovered from both *Scaphiopus spp.* and *R.catesbeiana* closely agree with the published descriptions of *C.complanatum*/*C.marginatum*. Although not included in Table 5.1, the metacercariae recovered from *S.bombifrons* were indistinguishable from those from *S.multiplicatus* and *R.catesbeiana*. A review of

the published literature indicates that host specificity and tegmental spine morphology may not provide clear distinctions between *C.attenuatum* and *C.complanatum*/*C.marginatum*. McAllister (1990) commented that 'caution should be exercised before arriving at an identification based solely on the traditional view of host specificity'. Feizullaev & Mirzoeva (1983) in their review of the genus, commented on the numerous characters used for identification '..it becomes clear that variability of these characters was conditioned by individual, age and ecophenotypic variations'. However, Larsen & Uglem (1990) cited work by Hunter & Hunter (1934 & 1935) relating to cross-feeding and cercarial infectivity experiments which supported two species of *Clinostomum*, one specific to frogs and one to fish. Further investigation is required to establish whether two species are present in the U.S.A. suggested by McAllister (1990) or if *C.attenuatum* is also a synonym of *C.complanatum* originally proposed by Feizullaev & Mirzoeva (1983).

Adult worms of this genus are generally endoparasites of the mouth and oesophagus of piscivorous birds (McAllister, 1990) but Prudhoe & Bray (1982) also list crocodilians as definitive hosts. Predators of anurans in the San Simon Valley which might represent final hosts include herons, raccoons, coyotes and snakes.

Almost continuous transmission of *C.complanatum* to *R.catesbeiana* is possible in permanent water-bodies and this would explain the high prevalence of infection recorded in this study. As there is no relationship between host SVL and infection,

the variation in intensity of *C.complanatum* infection in *R.catesbeiana* over the two sampling years is most likely a result of the smaller sample size in 1992. For the spadefoot toads, infection is restricted to the period of spawning, a maximum of 24 hours per annum (Tinsley, 1989). At Millers Pond, between 1991 and 1992, the prevalence of infection fell by over 50% but the mean intensity doubled. The over-representation of the *S.multiplicatus* genotype (57/65) in 1991 and the higher prevalence of *C.complanatum* in a proportionately larger sample of *S.bombifrons* in 1992 lead to equivalent levels of infection in consecutive years for the pooled samples. The constraints on transmission, compounded by sample size will have had a major influence in producing the poisson distribution of *C.complanatum* in *Scaphiopus spp.* compared to the positively skewed distribution recorded for *R.catesbeiana* (Fig.5.4). A more extensive survey may reveal that *C.complanatum* is more aggregated within *Scaphiopus spp.*. Whitfield (1979) noted that a limited sample from a particular group of hosts may produce a poisson distribution, however, if sampling is extended, the compounding of numerous poisson distributions would result in an overdispersed distribution.

Male *S.multiplicatus* spend the majority of their time in breeding ponds actively searching for mates, whilst male *S.bombifrons* ring the periphery of the pond (Simovich, 1985). Theoretically, *S.multiplicatus* males will be exposed to a greater number of infective stages, moving through a larger body of water. Male:female ratios were slightly higher for *S.bombifrons* (see Chapter 2). Table 5.2B shows that indeed *S.multiplicatus* harbour higher worm burdens, however, they form the majority of the specimens collected, and prevalences are lower than those recorded

for the limited samples of *S.bombifrons*. Therefore, the data presented do not allow any firm conclusions to be made on behaviourial traits between the two species of spadefoot toad and their influence on infection.

The absence of infected spadefoot toads in the vicinity of Willow Pond (pond A) may be a function of a small sample size, or more probably, due to predation: any toads entering this pond are likely to fall prey to the resident bullfrogs. *R.catesbeiana* has been present in Willow Pond for over 10 years; each year during the monsoons they emigrate from this site, commonly dispersing 5 to 6km by the end of the season. The majority probably die when the rains cease, but it is possible that these frogs may colonise new ponds (for example, those recovered from pond B). The range of *C.complanatum* infection documented in this study corresponds with the migration range of *R.catesbeiana*. However, this is also dependent upon the further establishment of snails and the foraging patterns of the definitive host(s), in conjunction with the human management of water resources that will have a major influence the future geographical distribution of infection.

The spadefoot toads examined in this study were collected on breeding nights, typically their first emergence of the year (Dimmitt & Ruibal, 1980). Therefore, they must have carried the majority of their infection for at least 10 months, from the previous breeding season. As 137 (94%) of the cysts recovered from *Scaphiopus spp.* during field dissections were active, this indicates the capacity of *C.complanatum* for prolonged survival. Other researchers have found similar examples of persistence; Ulmer (1970) cited Edney (1940) who reported that

metacercariae of *C.complanatum* remained viable for up to 45 months in catfish. Due to the potential longevity of metacercariae in *Scaphiopus spp.*, an extended longitudinal survey would be required to establish whether the infection of spadefoot toads was a chance, temporary event or a persistent occurrence.

Glucose uptake in digeneans is achieved by facilitated diffusion or active transport, depending upon the developmental stage of the fluke. *C.marginatum* was found to be unusual as both transport systems are expressed simultaneously in the metacercarial form (Uglem & Larsen, 1987; Uglem *et al.*, 1991). This ability may explain Oldaug's (1955) finding of metacercariae with an equivalent glycogen content when recovered from starved or fed *Rana pipiens*. This ability will not only help the metacercariae to survive host hibernation but also further deplete the finite resources of *Scaphiopus spp.*. This may explain the similar size of metacercariae from hosts which are active and feed throughout the year (*R.catesbeiana*) with those that hibernate for approximately 10 months per annum (*Scaphiopus spp.*).

Kagei *et al.* (1984) noted that fish infected with *C.complanatum* were deformed in comparison to uninfected controls. Lo *et al.* (1982) suggested that the metacercariae may cause lesions in host tissue, but only small fish suffered significant mortality. In this study, *Scaphiopus spp.* had a maximum intensity of 18 cysts (*R.catesbeiana* 114), with worms encysted throughout the body, but particularly cysts close to the glottis, jaw articulation and the eyes may impair function. For example, Etges (1991) reported an excysted metacercaria of

C.attenuatum moving within the eye of *B.marinus*.

Human infection in Japan has been reported by a number of authors (see Aohagi, Shibahara, & Kagota, 1993), with symptoms including pharyngeal pain, cough and blood-flecked sputum (Kagei *et al.*, 1984). The practice of eating raw fish in Asia has no doubt been a contributory factor, and so the consumption of inadequately cooked bullfrog legs in the U.S. should be considered a possible source of infection.

This is the first record of digenean infection of *S.bombifrons* and *S.multiplicatus* in the southwestern United States. Other endemic amphibians at risk in the study area include *Scaphiopus couchii*, *Bufo alvarius*, *B.cognatus* and *B.debilis*. Human management of the ecosystem has resulted in both faunal and habitat changes in the San Simon valley. If more permanent ponds are constructed, infection with *C.complanatum* may spread, as will the predation pressures exerted by *R.catesbeiana*.

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	A.			B.		
	Mean	Range	S.D.	Mean	Range	S.D.
Body length	6.28	4.90-7.54	0.75	5.59	4.09-6.79	0.71
Greatest width	1.94	1.46-2.57	0.28	1.96	1.61-2.28	0.22
O.S. length	0.30	0.25-0.34	0.02	0.32	0.28-0.36	0.02
O.S. width	0.37	0.32-0.44	0.03	0.40	0.34-0.48	0.04
V.S. length	0.86	0.72-1.24	0.08	1.01	0.81-1.14	0.10
V.S. width	0.89	0.79-1.06	0.08	1.03	0.88-1.14	0.08
Spine length	9µm	7-11µm	1.3µm	9µm	7-11µm	1.5µm

Table 5.1 Body dimensions of *Clinostomum complanatum* metacercariae recovered from **A.)** *Rana catesbeiana* and **B.)** *Scaphiopus multiplicatus*. 20 metacercariae measured from each host. (Abbreviations: O.S. = oral sucker; V.S. = ventral sucker). All measurements in mm, except where indicated.

A.

Host	Site(s)	Year	Prevalence	Intensity	R.density	n
Rana	Willow	1991	93.3	28.1	26.2	15
Rana	Willow	1992	100.0	9.0	9.0	5
Rana	Windmill	1991	100.0	31.5	31.5	2

B.

Host(s)	Site(s)	Year	Prevalence	Intensity	R.density	n
S.m.	Millers	1991	26.3	7.9	2.1	57
S.b.	Millers	1991	60.0	3.7	2.2	5
Hybrids	Millers	1991	0	0	0	3
S.m.	Millers	1992	11.1	14.0	1.6	9
S.b.	Millers	1992	36.4	8.3	3.0	11
S.m.	No.1	1991	14.3	3.0	0.4	7
S.b.	No.1	1991	0	0	0	8
Hybrids	No.1	1991	0	0	0	1
S.m.	Black Dog	1991	7.1	4.0	0.3	14
S.b.	Black Dog	1991	0	0	0	3
Hybrids	Black Dog	1991	0	0	0	2

C.

Host(s)	Site(s)	Year	Prevalence	Intensity	R.density	n
Rana	All Sites	1991	94.1	28.5	26.8	17
Rana	All Sites	1992	100.0	9.0	9.0	5
Scaph spp.	Millers	1991	27.7	8.0	2.2	65
Scaph spp.	Millers	1992	25.0	9.4	2.4	20
Scaph spp.	No.1	1991	6.3	3.0	0.2	16
Scaph spp.	Black Dog	1991	5.3	4.0	0.2	19
Scaph spp.	All Sites	1991	8.2	7.6	0.6	243
Scaph spp.	All Sites	1992	9.1	9.4	0.7	55

Table 5.2 The distribution of infection of *Clinostomum complanatum* in A.) *Rana catesbeiana*; B.) *Scaphiopus spp.* by genotype and C.) Summary of pooled host samples from study sites in the San Simon valley, Arizona/New Mexico, U.S.A.

(Abbreviations: *Rana* = *R.catesbeiana*; *S.b.* = *S.bombifrons*; *S.m.* = *S.multiplicatus*; Hybrids = all hybrid genotypes; *Scaph spp.* = pooled samples of *S.bombifrons*, *S.multiplicatus* and hybrids; Prevalence = prevalence expressed as a percentage; Intensity = mean intensity; R.density = relative density; n = sample size; Sites as listed in the text).

5.6 Legends.

Fig.5.1 *Clinostomum complanatum*, metacercaria recovered from *Scaphiopus multiplicatus*. **Abbreviations: gc**, gut caeca; **gp**, genital pore; **od**, oviduct; **os**, oral sucker; **ov**, ovary; **sm**, seminal vesicle; **ts**, testis; **vd**, vas deferens; **vs**, ventral sucker. Vitelline system not shown. **Scale bar:** 1 mm.

Fig.5.2 Metacercaria drawn in life (ventral perspective), **A.** Extended, indicating concave body form with oral sucker partially retracted into the muscular collar; **B.** Extremes of fore-body extension and contraction.
Scale bar: 1 mm.

Fig.5.3 Prevalence of *Clinostomum complanatum* from *Scaphiopus spp.* and *R.catesbeiana* from study sites sampled in 1991 in the San Simon Valley, Arizona/New Mexico, U.S.A. (Sites as described in Chapter 2, except: A = Willow pond; B = Windmill pond).

Fig.5.4 Frequency distribution of *Clinostomum complanatum* from **A.** *Rana catesbeiana* (n = 17) and **B.** *Scaphiopus spp.* (n = 243 [pooled sample]) from study sites sampled in 1991 in the San Simon Valley, Arizona/New Mexico, U.S.A.

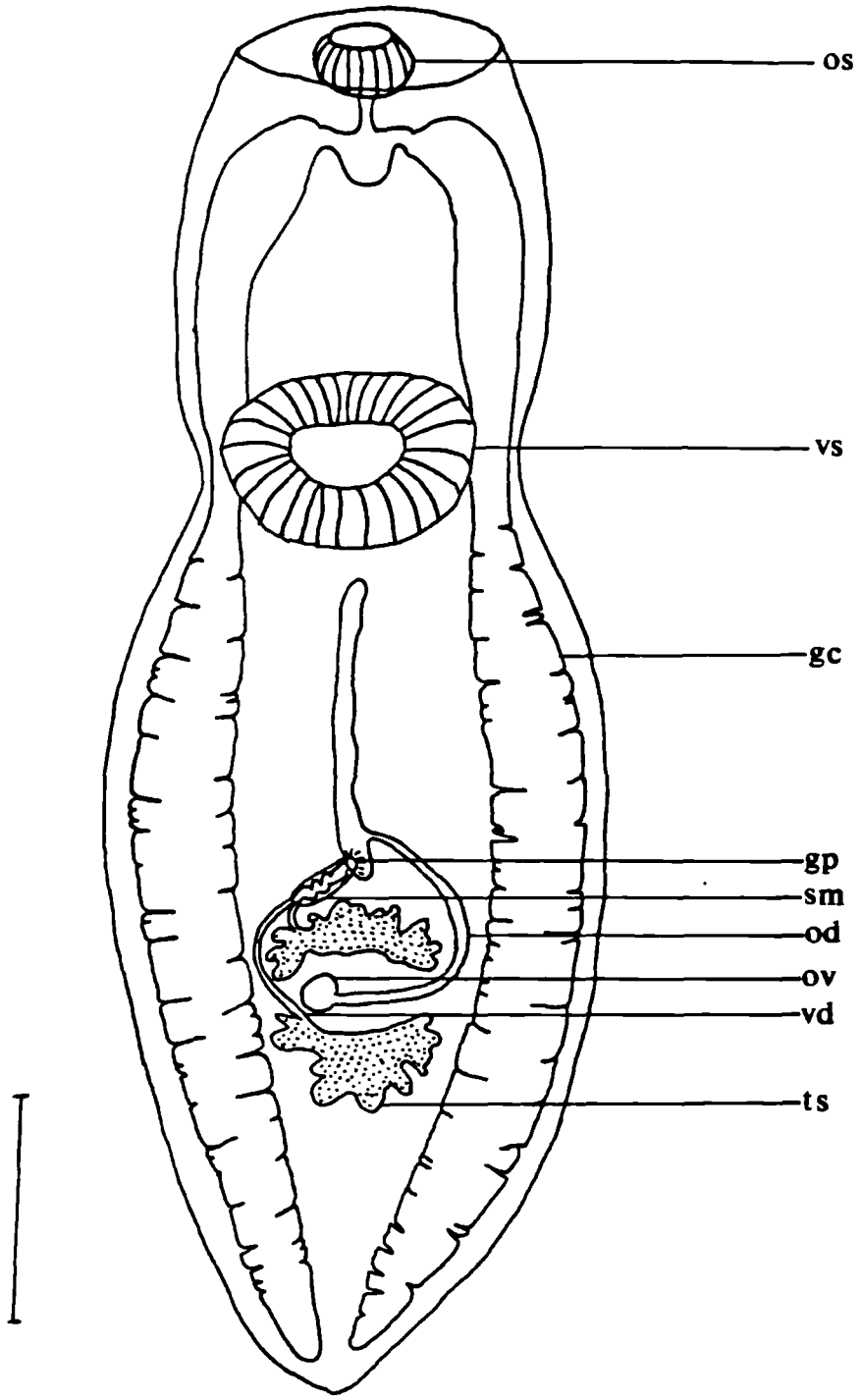


Fig.5.1

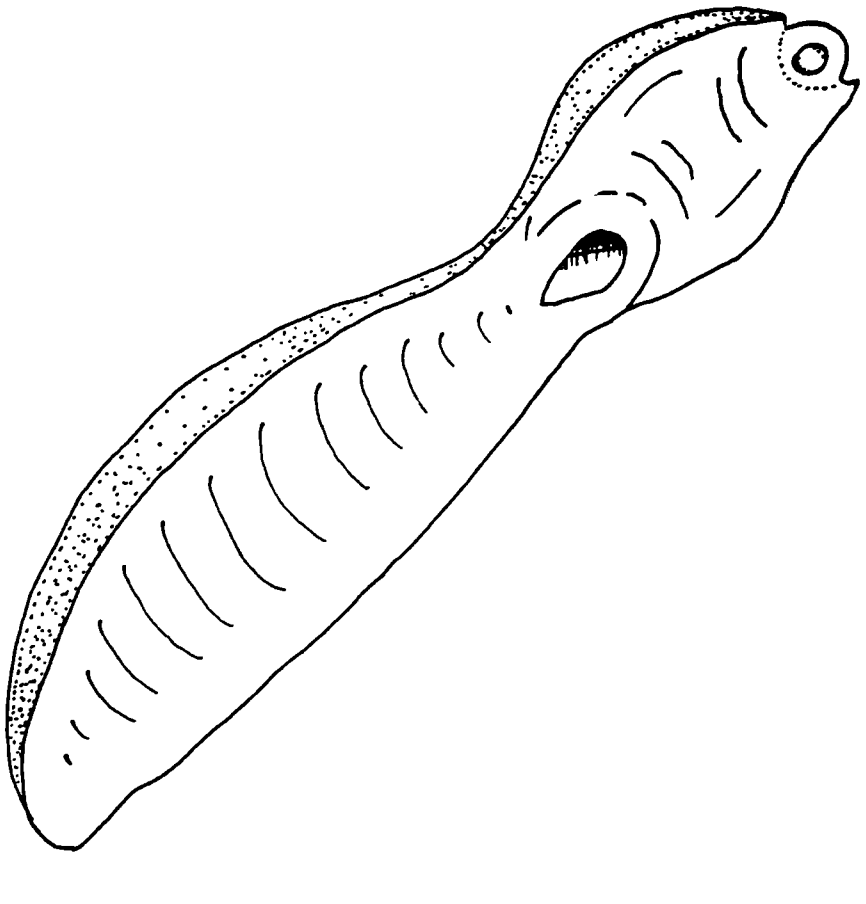
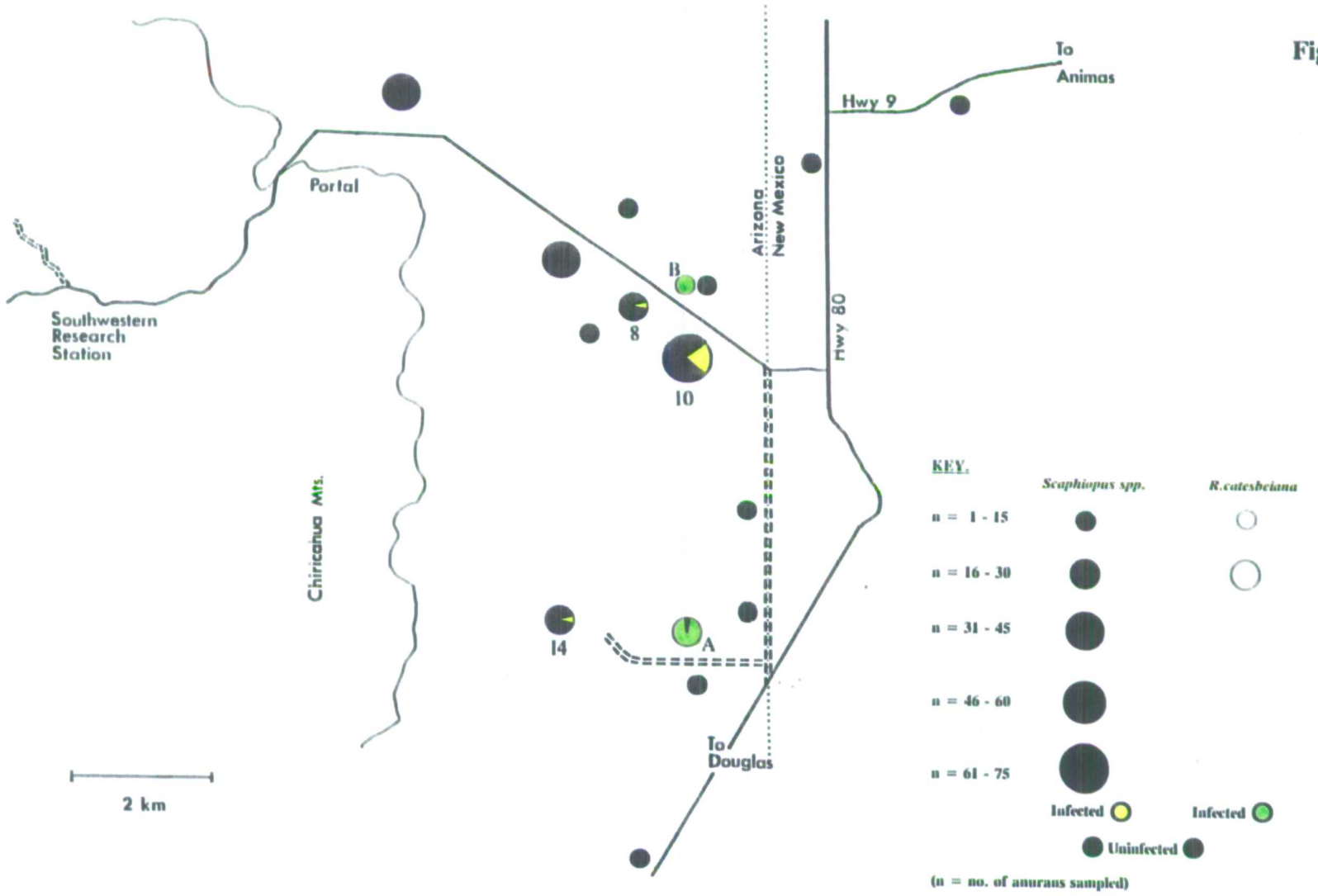
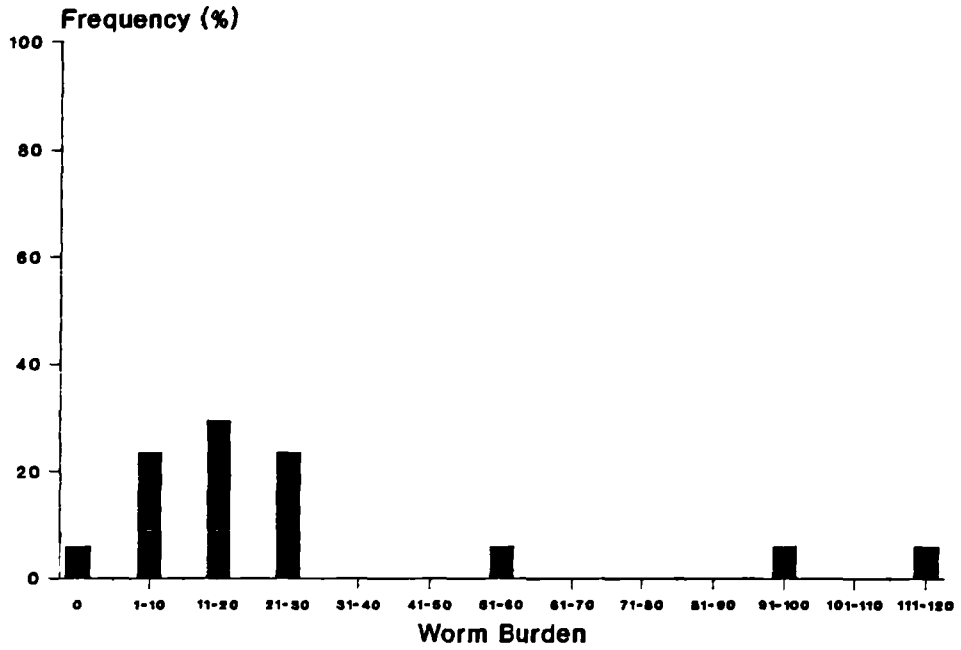


Fig.5.2

Fig.5.3


To Animas
Hwy 9
Portal
Arizona
New Mexico
Hwy 80
Southwestern Research Station
B
8
10
Chiricahua Mts.
14
A
To Douglas
2 km
KEY.
Scaphiopus spp.
R.catesbeiana
n = 1 - 15
n = 16 - 30
n = 31 - 45
n = 46 - 60
n = 61 - 75
Infected
Infected
Uninfected
(n = no. of anurans sampled)

A


Frequency (%)
100
80
60
40
20
0
0
1-10
11-20
21-30
31-40
41-50
51-60
61-70
71-80
81-90
91-100
101-110
111-120
Worm Burden

B

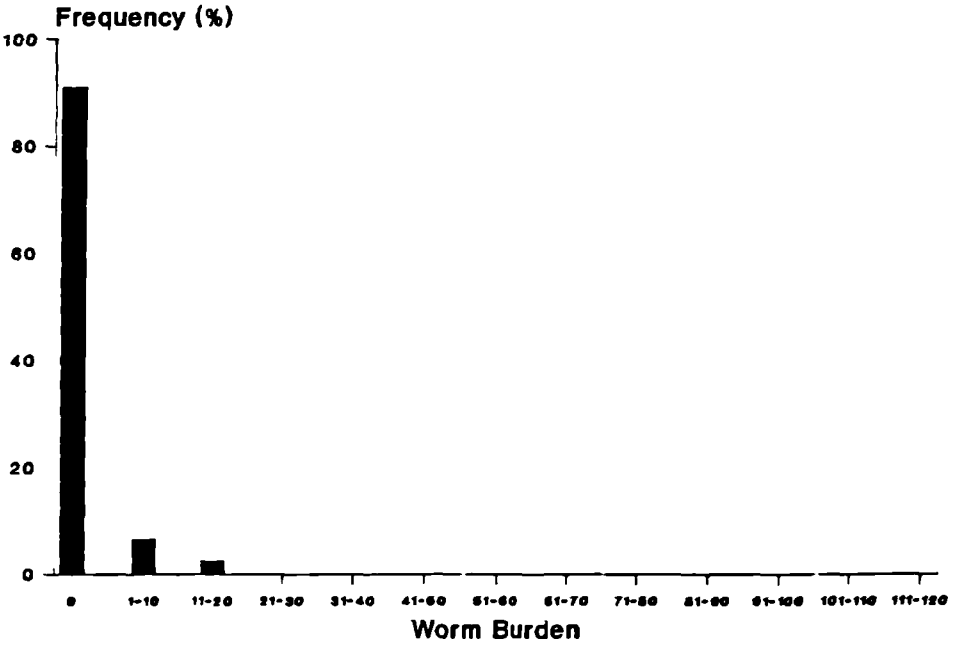

Frequency (%)
100
80
60
40
20
0
Worm Burden

Fig.5.4

Chapter 6.

Polystoma nearcticum (Paul, 1935) Price, 1939 (Monogenea:Polystomatidae): parasitic in the diploid-tetraploid species complex of *Hyla chrysoscelis* and *Hyla versicolor* (Anura:Hylidae) in North America.

6.1 Abstract.

This chapter presents the first record of *Polystoma nearcticum* (Paul, 1935) Price 1939 from the diploid Gray treefrog, *Hyla chrysoscelis*. This monogenean has previously been reported from *H.cinerea*, *H.squirella* and the tetraploid Gray treefrog, *H.versicolor*. A total of 182 Gray treefrogs were examined for polystomatid infection and morphological analyses of 14 *Polystoma* recovered from *H.chrysoscelis* and 42 from *H.versicolor* were undertaken. Infection levels were comparable at the major sympatric/syntopic site from which collections were made, *H.chrysoscelis*: prevalence = 28.6%, mean intensity = 1.75, relative density = 0.5; and *H.versicolor*: prev. = 29.4%, int.= 1.2, r.d. = 0.35. Preliminary experimental cross-infections suggested that branchial worms develop on heterospecific hosts with an equivalent growth rate to conspecific infections. The data gathered indicate that a single taxon, *P.nearcticum*, infects both members of this polyploid complex. The geographical range of *P.nearcticum* was greatly extended with records from Florida, Louisiana, Minnesota, Missouri and Texas from 3 of the 5 documented host lineages.

6.2 Introduction.

The North American Gray treefrogs are native to the woodlands of the mid-west and eastern United States. They form a cryptic species pair, distinguished by mating call and chromosome number, *Hyla chrysoscelis* is the diploid progenitor (2n = 24) of the tetraploid *Hyla versicolor* (2n = 48) (Wasserman, 1970). The origins of the *H.chrysoscelis/H.versicolor* complex have been the subject of detailed and varied research. Examination and differentiation of this complex has

involved karyotypic analysis (Bogart & Wasserman, 1972), cytochemistry (Bachmann & Bogart, 1975), albumin immunology (Maxson, Pepper & Maxson, 1977), geographic variation in mating call (Ralin, 1977), morphology (Ralin & Rogers, 1979; Matson 1990), rRNA sequence divergence (Romano & Vaughn, 1986), electrophoresis (Ralin & Selander, 1979; Ralin, Romano & Kilpatrick, 1983) and mtDNA analysis (Ptacek, Gerhardt & Sage, 1993). Although a number of hypotheses have been proposed, Ptacek *et al.* (1993) suggested that there have been three independent autopolyploid origins of the tetraploid from *H.chrysoscelis*, which in turn has two distinct eastern and western lineages. It is interesting to note that in areas of sympatry, two of the *H.versicolor* lineages are in secondary contact with *H.chrysoscelis* (i.e. they are not sympatric with the lineage from which they arose). No maternal ancestor for the third tetraploid lineage has been recorded. Reproductive isolation is maintained by female selection: Gerhardt *et al.* (1994) found that mis-matings are rare, estimating that hybrids constitute approximately 0.05% of sympatric populations.

The anatomy of the genus *Polystoma* was documented by Zeller (1872, 1876) and Gallien (1935). Paul (1935) first recorded *Polystoma integerrimum nearcticum* from *H.versicolor*, subsequently producing a full description of adult and branchial forms from *H.versicolor* and *H.cinerea* collected in Connecticut State, U.S.A. (Paul, 1938). Price (1939) added *H.squirella* to the list of hosts and elevated the status of *Polystoma integerrimum nearcticum* to that of a species in its own right, *P.nearcticum*.

In addition to *P.nearcticum*, a further 11 species of *Polystoma* have been recorded from the New World: *P.andinum*, *P.borellii*, *P.cuvieri*, *P.diptychi*, *P.guevarai*, *P.lopezromani*, *P.naevius*, *P.napoensis*, *P.praecox*, *P.stelli* and *P.touzeti* by a number of authors (Caballero & Cerecero, 1941; Combes & Laurent, 1974, 1978, 1979; Vigueras, 1955; Vaucher, 1986, 1987, 1990). No detailed reviews of anuran polystomatids occurring in nearctic or neotropical regions have been made, typically with only species and locality records presented in the literature.

Research into the systematics of the *Polystoma* has centred on Africa, with a number of authors evaluating the characters that are the most applicable for differentiation between species (Maeder, 1973; Tinsley, 1974; Combes & Channing, 1978-1979; Murith, 1981, 1982; Kok & van Wyk, 1986; Van Niekerk, 1992). These works have been the subject of much debate and provide a detailed background to some of the theoretical concepts within the group. Strict host specificity has been proposed for anuran polystomatids (Bourgat, 1977; Bourgat & Salami-Cadoux, 1976; Combes & Channing, 1978-1979; Euzet, Combes and Knoepffler, 1966; Murith, 1981). However, Prudhoe & Bray (1982) questioned the validity of species differentiation based mainly on host specificity. There have been a number of examples in which polystomatids have more than one host species (Murith, 1981; Vaucher, 1990) or may occur syntopically (Bourgat & Murith, 1980; DuPreez & Kok, 1992).

Polystoma africanum was the first species of this genus to be described from

Africa (Szidat, 1932). Subsequently, other species were described but it was Euzet *et al.* (1966) who first suggested strict host-specificity within the group, by describing two sub-species of *P.africanum* recovered from different host species. Their division was supported by differing hamulus morphology. Combes (1966, 1968) found that for European *Polystoma*, oncomiracidia exhibited a preference when given a choice of host species. Furthermore, Bourgat & Salami-Cadoux (1976) and DuPreez & Kok (1993) referred to unpublished cross-infection experiments involving African *Polystoma* which document unsuccessful oncomiracidial establishment on heterospecific hosts. However, it should be noted that, Kok & DuPreez (1987) successfully established *P.australis* on a number of amphibian genera, with neotenics reaching maturity. In nature, the same authors recovered *P.australis* from alternative host tadpoles. Maeder (1973) recorded five species of neotenic and two species of pre-adult *Polystoma* from 2 and 3 tadpole species respectively. The work of Combes (1966, 1968) in demonstrating host-specificity in European *Polystoma* provided the basis for Maeder, Euzet & Combes (1970) to elevate those species described by Euzet *et al.*, (1966) to species level, in addition to four other species of *Polystoma*. Maeder *et al.* (1970) based their divisions on '..general proportions, the shape of the hamuli and by the outline of the gut', but the need for experimental confirmation was noted. A further 2 new species of *Polystoma* were described by Maeder (1973).

The first major review of the systematics of African polystomatids was by Tinsley (1974) who proposed a '*Polystoma africanum* species complex'. Whilst maintaining the status of 13 of the species described, Tinsley (1974) noted the

morphological similarities between the species and that the divisions between them on the basis of specificity and morphology were not as clear as originally proposed. Combes & Channing (1978-1979) sub-divided the '*Polystoma africanum* species complex' of Tinsley (1974) into three, based on intestinal morphology. This new grouping contained 17 of the 21 African *Polystoma* species described at that time.

Murith (1981) suggested that chaetotaxy and the morphology of the marginal hooklets provided useful diagnostic tools. Murith (1981) in an extensive review, placed African polystomatids into two groups; the '*africanum*' and '*togoensis*' complexes. The basis for differentiation was the ratio of total length of marginal hooklet I against the handle length, which could be applied to juvenile specimens. Both re-assessments of the original grouping by Tinsley (1974) omitted some previously described species without explanation. Since 1981 the majority of descriptions have come from Kok and co-workers (DuPreez & Kok, 1992 & 1993; Kok & van Wyk, 1986; Kok & Seaman, 1987; Van Niekerk, Kok & Seaman, 1993). This work has been based on *Polystoma* recovered in South Africa, following the principles proposed by Murith (1981). There are only two keys existing for the identification of polystomatids. The first was by Saoud (1967), but this was limited to only a few species. Prudhoe & Bray (1982) provided a generic key but omitted *Metapolystoma* Combes, 1976.

Van Niekerk (1992) produced an extensive review and multivariate analysis of the relationships of African polystomatids. It was found that a few species were

easily distinguished, mainly by the possession of unique characters (i.e. *P.mashoni* does not possess intestinal caeca which unite in the posterior of the worm). The remaining species often had a similar host distribution and were difficult to separate on a morphological basis alone. For example, *P.lamottei*, *P.makereri* and *P.ragnari* were unique by their possession of a network of intestinal caeca, furthermore *P.makereri* and *P.ragnari* infect the same host genus in forest habitats. In addition, *P.lamottei* was described from a host which the authors considered to be accidentally infected (Bourgat & Murith, 1980). This led Van Niekerk (1992) to suggest that *P.lamottei*, *P.makereri* and *P.ragnari* may indeed be a single species. A similar proposal for synonymy was made for *P.africanum*, *P.pricei* and *P.togoensis*, in addition to *P.dorsalis* and *P.llewellyni*. It is clear that without further experimental work the delineation of *Polystoma* species will be problematic.

In contrast to the relatively extensive documentation of Old World anuran polystomatids, there have been few studies of North American *Polystoma*. As noted above, *Polystoma nearcticum* has only previously been reported from *H.cinerea*, *H.squirella* and *H.versicolor*. If *H.chrysoscelis* is infected with a polystome then does the generalist *P.nearcticum* infect this host species or does *H.chrysoscelis* harbour its own distinct species-specific monogenean? Furthermore, how widely and within which of the two *H.chrysoscelis* and three *H.versicolor* lineages does this monogenean occur?

6.3 Materials and Methods.

6.3.1 Collection of *Hyla chrysoscelis* and *H.versicolor*.

Collections were made of both species of Gray treefrog, 92 specimens of *H.chrysoscelis* and 90 *H.versicolor*. Specimens collected at sites 1-3 (see below) were made by the author and R.C.Tinsley, the remaining samples (sites 4-12) were supplied by Prof. H.C.Gerhardt. Individual males were readily located by their loud trill call and were captured by hand. Calling sites were typically close to the water in the lower branches of trees, shrubs and undergrowth. Females do not call and so unless already in close proximity to a calling male or in amplexus, they were only encountered by chance. Each frog was either put in an individual container or an aquarium with up to 15 frogs in total. Both types of container were filled with approximately 2 cm of aged water. In areas of sympatry, the two species were distinguished on the basis of mating call for males and by flow cytometry (as described by Gerhardt *et al.*, 1994) for single females and amplexed pairs. Treefrogs possess nucleated red blood cells, consequently flow cytometry provides an effective method of species identification.

The collection sites are mapped in Fig.6.1 and listed below with species sample size per site in brackets.

1). Boone Co., Missouri. Ashlands Reserve 3km E of Rt. 63; allopatry *H.versicolor* (n = 60).

2). Phelps Co., Missouri; Rt.K, 3.8km W of the jct. of Rt. ZZ (6.5km W of Beulah); sympatry *H.chrysoscelis* (n = 17) & *H.versicolor* (n = 14).

3). Barry Co., Missouri; Rt. TT 4.8km E Jct. Hwy 39 (10km S of Aurora); sympatry *H.chrysoscelis* (n = 13) & *H.versicolor* (n = 3).

4). Bastrop Co., Texas; Hwy 71, 0.5km E jct. Cottletown Rd; sympatry *H.chrysoscelis* (n = 4), no *H.versicolor* collected.

5). Wood Co., Texas; U.S. 80, 3.2km E of Hoard; sympatry *H.chrysoscelis* (n = 4) & *H.versicolor* (n = 5).

6). Leon Co., Florida; NW edge of Tallahassee; *H.chrysoscelis* allopatry (n = 27).

7). Otter Trail Co., Minnesota; 8km S of Dent; sympatry *H.chrysoscelis* (n = 5) & *H.versicolor* (n = 5).

8). Ottawa Co., Oklahoma; edge of Wynadotte; sympatry *H.chrysoscelis* (n = 3) & *H.versicolor* (n = 3).

9). Ballard Co., Kentucky; 1.6km SW of Wickliffe; allopatry *H.chrysoscelis* (n = 7).

10). St. Martin Parish, Louisiana; Hwy. 353, 5.8km E jct. Hwy. 94; allopatry *H.chrysoscelis* (n = 1).

11). Baltimore Co., Maryland; edge of Townson; allopatry *H.chrysoscelis* (n = 9).

12). Lancaster Co., Nebraska; edge of Lincoln; allopatry *H.chrysoscelis* (n = 2).

Frogs were dissected as described in Chapter 3. Those collected from sites 1, 2, 3, 7, 8, 9, 10 and 11 were dissected within a few days of capture. The sample taken from site 12 was maintained in captivity for 9 days before necropsy. Frogs

from sites 4, 5 and 6 were fixed in formalin the day after capture and transferred to 70% alcohol one week later. *Polystoma* recovered from the urinary bladder of freshly dissected specimens were fixed in a 10% formal saline solution, under 22x22mm coverslips, with sufficient pressure to display the hamulus profile and gut branches.

6.3.2 **Cross-infection experiments.**

Upon return to the laboratory, water from the holding containers and 3 further washings were collected into crystallising dishes. Polystomatid eggs (released with host urine) were allowed to settle to the bottom before the excess water was decanted. The eggs were concentrated into the centre by swirling the final volume of water. These were drawn into a pasteur pipette and released into 5cm diameter petri dishes and maintained at 20.6+_ 0.8^{0}C.

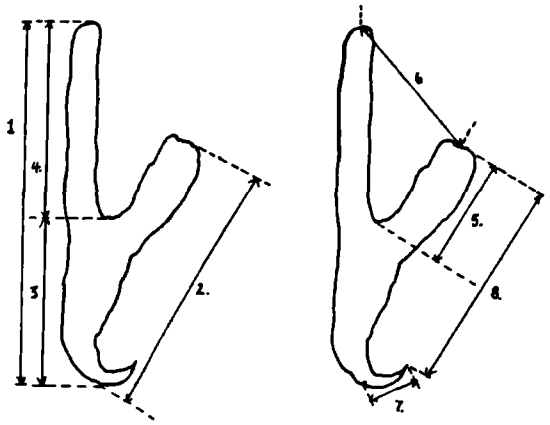
All cross-infections were undertaken using eggs derived from allopatric populations of both *H.chrysoscelis* and *H.versicolor*. Oncomiracidia hatched under standard conditions and were utilised within 1h of emergence. Only those oncomiracidia which swam vigorously were used for infection procedures. Single tadpoles (aged 1-2 weeks post-hatch) were exposed to 10 oncomiracidia in 5cm diameter petri dishes containing 15ml of aged water. Oncomiracidia were gently taken up into a pasteur pipette and released into the centre of the infection dish with the minimum current. All oncomiracidia were counted, under a binocular microscope, as they entered the infection dish. After 24h the tadpoles were moved to larger containers (plastic drinking cups) for easier care.

For each cross-infection and control group, sub-samples were dissected at 2 day intervals post-infection (p.i.). The dissection of tadpoles was undertaken after anaesthetisation in a 1% MS 222 solution (Sandox Chemical Co.). This concentration rapidly killed the tadpoles but the worms remained attached and mobile. Tadpoles had excess moisture removed on tissue paper prior to weighing (to nearest 0.001g) on a top-pan balance. The nose to tail-tip length (to nearest 0.1mm) was also recorded. The tadpole was rinsed and put in a 5cm diameter petri dish filled with aged tap water. The epidermis was pulled away exposing the gills which were examined *in situ*. Each gill was then removed and examined separately. The branchial chamber and surrounding water were checked for any worms that became detached during dissection. The sites of attachment were recorded and all worms were fixed in a 10% formal saline solution under 18x18mm coverslips with sufficient pressure to display the hamulus profile.

On return to Q.M.W. the worms were stained and mounted *in toto* for morphological analysis using a Nikon Optiphot UFX IIA microscope (as described in Chapter 3). Measurements of length and width (in mm) were taken for the body, haptor, pharynx, ovary, oral and haptoral suckers for both adult and branchial forms. In addition, genital spine length, hamulus length and the body width at the vaginae was measured for the adult worms.

Hamulus morphology was recorded by camera lucida drawings of specimens mounted *in toto*. The length of the hamulus blade was measured with a curvimeter (constructed from graph paper - Chartwell) from drawings made at x100

magnification. Further analyses used whole hamulus drawings made at x20 magnification. For each hamulus eight parameters were scored, (1) total length, (2) guard length, (3) tip to notch, (4) notch to handle, (5) notch to guard, (6) guard to handle, (7) point length and (8) point to guard. For reference, the exact points are shown below.



6.4 Results.

6.4.1 **Infection levels.**

Polystoma was recovered at Boone Co., Missouri (site 1); Phelps Co., Missouri (site 2); Bastrop Co., Texas (site 4); Leon Co., Florida (site 6); Otter trail, Minnesota (site 7) and St. Martins Parish, Louisiana (site 10). The prevalence of polystomatid infection ranged from 20-100% at intensities of 1.0-5.0 parasites/host between these 6 sites (Table 6.1), although it should be noted that the upper extremes were recorded from the single host at site 10. Overall, at 3 of the collection sites sample sizes were 5 or less, therefore, further analyses were restricted to the 3 remaining locations. Frequency distributions of *Polystoma* in the Gray treefrogs at these sites (Fig.6.2) indicate that in the sample from Florida (site 6), 4 individuals harbour over 70% of the worms recovered, consequently these frogs have a strong influence on the intensity of infection. The sample size from site 1 is reduced from that stated in the Method, as 14 frogs were not part of a

random sample. However, the worms they harboured have been used in the morphological analyses. The plots from site 2 are from Phelps, Missouri, which was a sympatric and syntopic pond. Both have similar frequency distributions and have comparable infection levels: *H.chrysoscelis* (prev. = 28.6%, int. = 1.75 & r.d. = 0.5) and *H.versicolor* (prev. = 29.4%, intensity = 1.2 & r.d. = 0.35).

6.4.2 Description of *Polystoma nearcticum* from the urinary bladder.

The description and measurements of the adult form are based solely on 42 sexually mature, egg producing specimens recovered from the urinary bladder of *H.versicolor* at burdens of 1-4 parasites/host. No immature parasites were found in adult hosts. The measurement of the larval sclerites was based on larvae hatched from eggs laid by the adults as well as the adult specimens themselves. Measurements are in mm with the range, mean and standard deviation recorded (except where indicated). A summary of the main characters is recorded in Table 6.2, which also incorporates data relating to the polystomatid recovered from *H.chrysoscelis*.

The overall body form (Fig.6.3) is lanceolate with a terminal opisthaptor. The parasite measures 2.69-8.85 (6.12 +_ 1.17) in total body length with a maximum width of 0.91-3.00 (2.30 +_ 0.50). The haptor ranges from 0.71 to 1.54 (1.11 +_ 0.19) in length from 1.15 to 2.25 (1.82 +_ 0.27) in width. The overall haptor length to total body length ratio is 0.18.

6.4.2.1 **Haptor.**

The haptor bears three pairs of muscular suckers with a single pair of hamuli situated between the first sucker pair. Marginal hooklet I measures 17.6-23.5μm (20.4 +_ 1.6μm) in length, with a total length to handle length ratio of 1.98. Hamulus length measures 0.25-0.42 (0.35 +_ 0.04) with a mean 1/2 ratio of 1.19 (Table 6.3). During branchial development the three sucker pairs exhibit a differential rate of development (see Chapter 7), this is not observed in the adult. The posterior pair of suckers measure 0.23-0.40 (0.32 +_ 0.04) in diameter, the middle pair range from 0.21 to 0.40 (0.34 +_ 0.04) and the anterior pair measure 0.22-0.41 (0.33 +_ 0.04). Pooled data for the three pairs give an overall sucker diameter of 0.22-0.40 (0.32 +_ 0.04).

6.4.2.2 **Intestinal arrangement.**

The pyriform muscular pharynx lies just behind the mouth at the anterior of the body. Pharyngeal length ranges from 0.17 to 0.37 (0.28 +_ 0.05) and in width from 0.14 to 0.28 (0.21 +_ 0.03). The intestine bifurcates just behind the pharynx and possesses numerous branched and unbranched lateral and median diverticula. Diverticula may extend deep into the haptor. Anastomoses are formed posterior to the reproductive system and extend into the haptor, with no clear pattern.

6.4.2.3 **Reproductive system.**

The overall structure of the reproductive system is as described by Paul (1938). The ovary is comma shaped, more frequently on the right side of the body (23:19). The ovary is situated in the anterior third of the body and measures 0.13-

0.96 (0.69 +_ 0.17) in length and 0.07-0.53 (0.37 +_ 0.09) in width. The testis is multilobulate lying posterior to the ovary. The genital pore lies in a median position, slightly anterior to the level of the vaginae. The genital pore typically bears 8 spines (range 7-9) with a mean length of 30.2μm (+_2.9μm). Vitellaria are present throughout the body with collecting ducts originating from both sides of the body. A genito-intestinal canal lies on the ovarian side. Vaginae are present and lie immediately behind the pharynx. Eggs are stored in the short, convoluted uterus prior to release. The eggs measure 0.300 (+_0.025) in length and 0.150 (+_0.012) in width.

Gross hamulus morphology is consistent between *Polystoma* recovered from both hosts at the same locality (Fig.6.4) but there is some degree of geographical variation within *H.chrysoscelis* derived worms (Fig.6.5). The relationship between total hamulus length and mean blade length (Fig.6.6) indicates no separation between worms recovered from both host species. The analyses of the 8 hamulus measurements are summarised in Table 6.3. By comparing the ratios of the 8 parameters between *H.chrysoscelis* and *H.versicolor* derived worms, both have comparable means and standard deviation. However, the larger sample (*H.versicolor*) commonly has the greater range. If the total length of larval hooklet I (b) is plotted against the length between the handle to bifurcation point of the guard (a) there is no separation between the samples by species (Fig.6.8).

6.4.3 Developmental rate of cross-infections and controls.

The developmental rate of neotenic *P.nearcticum* used in conspecific infections of *H.versicolor* is documented in Chapter 7 and provides a basis for comparisons with the cross-infections in this study. With the limited numbers of *H.chrysoscelis* derived oncomiracidia available, the tadpole sampling regime was based mainly upon the recovery of representative worms at each age class rather than population/invasion success criteria. In addition to the worms described in Chapter 7, the following combinations were recovered:

1). *H.versicolor* derived oncomiracidia -> *H.chrysoscelis* tadpoles: 51 worms from 43 hosts.

2). *H.chrysoscelis* derived oncomiracidia -> *H.chrysoscelis* tadpoles: 19 worms from 17 hosts.

3). *H.chrysoscelis* derived oncomiracidia -> *H.versicolor* tadpoles: 13 worms from 23 hosts.

As total body length, haptor length, pharynx length and sucker diameter had low coefficients of variation for the *H.versicolor* controls (see Chapter 7), the same characters were chosen for comparison in this chapter. In addition, mean body length for branchial *P.nearcticum* was shown to be influenced by worm burden, consequently in Chapter 7, data was separated into low (n = 1-5) and high (n = 6-10) worm burdens. However, for this study, burdens of n = 1-10 were considered as only a limited number of infections were possible. Due to time restrictions in the field, representative worms could only be recovered up to 14

days p.i.

Controls and cross-infections have been divided into 4 groups for graphical representation in Figs. 6.8, 6.9 & 6.10: A) *H.versicolor* oncomiracidia -> *H.versicolor* tadpoles; B) *H.versicolor* -> *H.chrysoscelis*; C) *H.chrysoscelis* -> *H.chrysoscelis* and D) *H.chrysoscelis* -> *H.versicolor*. The representative body dimensions of branchial *P.nearcticum* over the study period are recorded in Figs. 6.8, 6.9 and 6.10. The primary finding from the limited number of infections, is that to 14 days p.i., *P.nearcticum* can establish and develop on heterospecific hosts. The largest sample of worms was recovered from the heterospecific crosses involving *H.versicolor* -> *H.chrysoscelis* (group B), from which it is apparent that, for all four parameters, the worms consistently increased in size over the experimental period. In the other groupings, major fluctuations were recorded for both cross-infections and controls, suggesting that intra-specific competition between worms and histological procedures may have been responsible. For example, in group C (*H.chrysoscelis* controls) there was a marked decline at 12 days p.i. which should be noted in context of worm burden. At 10 days p.i. worms were recovered from a tadpole harbouring 2 parasites, at 12 days p.i. 6 parasites/host and 14 days p.i. 2 tadpoles both harbouring 3 parasites/host. As an inverse relationship between mean body length and worm burden has been established (see Chapter 7), it is probable that the fluctuation is related to infrapopulation density. There was also a marked decline for group D (*H.chrysoscelis* -> *H.versicolor*) after 10 days p.i., however, the 2 specimens recovered after this time contracted during fixation. In both cross-infections and

controls, the ovary began as a darkly staining region at 6 days p.i. reaching its distinctive rectangular form by 14 days p.i. The timing of the appearance of haptoral suckers exhibited slight variation between groups, although no distinctive pattern was evident. The period from infection to the clear differentiation of each sucker pair is summarised in Table 6.4.

6.5 Discussion.

The morphology of the adult bladder form of the polystomatid monogenean recovered from both *H.chrysoscelis* and *H.versicolor*, is in agreement with the description of *Polystoma nearcticum* recorded by Paul (1938). The specimens recorded in this study display a greater range of sizes than those documented by Paul (1938), which is most probably due to histological methods and the larger sample size. Within this sample the degree of variation is greater for those worms recovered from *H.versicolor* (see Table 6.2), which again may be due to the larger sample size. In addition to those characters previously described (Paul, 1938), extra morphological features have been considered (width at vaginae, HL/L ratio, oral sucker length & width, cirrus spine length, ovary width, haptoral sucker diameter, marginal hooklet dimensions and hamulus dimensions). There is a clear partition between the blade and the main shaft of the hamulus in *P.nearcticum*, which facilitates accurate measurements of blade length. Vaucher (1990) proposed a species-specific relationship between total hamulus length and mean blade length/area for South American *Polystoma*. However, it is clear that on the basis of this analysis (Fig 6.6) there is no segregation between the worms recovered from *H.chrysoscelis* or *H.versicolor*. Although some variation in gross hamulus

morphology was observed between collection sites (Fig.6.5), this does not appear to be host specific, as in sympatric and syntopic localities hamuli from the worms recovered from both diploid and tetraploid hosts are comparable (Fig 6.4). Comparisons of the 8 hamulus measurements summarised in Table 6.3 also demonstrate little variation between host species. Further collections of both species of Gray treefrog from a number of sites are required in order to undertake a detailed analysis of any possible geographical heterogeneity. However, it should be noted that Van Niekerk (1992) concluded '..the hamuli cannot serve as a reliable character to distinguish between different *Polystoma* species'.

Murith (1981) documented species-specific hooklet I morphology in African anuran polystomatids and this has been supported by Kok & Seaman (1987), DuPreez & Kok (1992, 1993), Van Niekerk (1992) and Van Niekerk *et al.* (1993). This technique does not however separate the worms recovered from the two host species in this study (Fig 6.6). Once again further collections are required to assess whether geographic variation occurs. The analyses of sclerite morphology which have been shown to be species specific for other anuran polystomatids do not indicate any separation between the worms collected from *H.chrysoscelis* and *H.versicolor*, suggesting that *P.nearcticum* does infect both species of Gray treefrog. As speciation of the host complex is thought to have involved autopolyploidy, *P.nearcticum* will have been presented with the doubling of essentially the same genome, rather than fundamentally different genes. Consequently, further investigation is required before the provisional records of *P.nearcticum* from *H.cinerea* and *H.squirella* can be confirmed.

As it was only possible to set up limited cross-infection experiments over a 14 day period, only 64 worms were recovered from heterospecific hosts in a variety of worm burdens (1-10 parasites/host) which infected tadpoles of varying body weight (albeit of similar age). There are a number of variables which could not be moderated, particularly the range of infrapopulation densities, which (on conspecific hosts) have been shown to affect body size significantly (see Chapter 7). The fluctuations in size of the controls and cross-infections (Figs. 6.8-6.10) may be explained by these variables and contraction during fixation. However, it is clear that, to 14 days p.i., oncomiracidia can establish and grow on alternative host tadpoles. More extensive infection experiments are required to evaluate whether larvae of *P.nearcticum* infecting heterospecific hosts can reach neotenic maturity and/or successfully migrate and achieve sexual maturity in the urinary bladder. Kok & DuPreez (1987) found no preference in their limited cross-infections of *P.australis* between two anuran species, or in nature, where the hosts were sympatric. However, the same authors have reported other African species of *Polystoma* which do not successfully establish on other host species (DuPreez & Kok, 1993). As described in Chapter 3, the cross-infections of *Neodiplorchis scaphiopodis* resulted in the recovery of sexually mature worms from heterospecific hosts, confirming that a single species infects *S.bombifrons* and *S.multiplicatus*. Experimental confirmation of this nature was not possible for *P.nearcticum* because the sexual maturity of the worm is closely linked to that of its host, at 1 to 2 years.

The broad variation in infection levels recorded in Table 6.1 may be attributed to

the restricted host sample size at 3 of the 6 sites. However, it is important to note that in the larger samples, particularly at the sympatric and syntopic pond (site 2), infection levels were comparable for both host species. Furthermore, individual Mann-Whitney tests between sites 1, 2 and 6 (documented in Chapter 8) indicate that there are no significant differences of *P.nearcticum* distribution between host species or locality.

Ptacek *et al.* (1993) proposed that not only are there 3 separate lineages of *H.versicolor* but there are also two distinct, eastern and western lineages of *H.chrysoscelis*. The distribution of the tetraploids involves secondary contact with their diploid progenitors, except for the third (northwestern) lineage for which no maternal ancestor has been discovered. This study has recorded *P.nearcticum* from both eastern (Florida, Louisiana & Missouri) and western (Texas) lineages of *H.chrysoscelis* but only the northwestern lineage of *H.versicolor* (Minnesota & Missouri). Paul (1938) first described *P.nearcticum* from *H.versicolor* in Connecticut State which falls within the range of the eastern lineage. Therefore, *P.nearcticum* has been recorded from all Gray treefrog lineages except for the southwestern *H.versicolor* and possibly the undiscovered diploid ancestor of the northwestern *H.versicolor*. Further examination of the morphology of worms within each of the clades is required to find whether any differences have occurred since divergence. Vaucher (1990) suggested that the geographical distribution of *Polystoma* is typically narrower than the range of its particular host. Although a wider survey would be required, in addition to the record by Paul (1938) this study has recovered *P.nearcticum* from a range of sites, some close the maximum

extent of the hosts distribution.

As noted in the Introduction, only 12 *Polystoma* species have been recorded from the New World, with *P.nearcticum* being the single representative from North America. The closest species geographically are *P.naevius* from Mexico and *P.stelli* from Cuba. *P.nearcticum* is distinct from *P.naevius* in a number of features: the latter is smaller; has a differing gut and egg morphology; has a larger guard on a smaller hamulus, and finally the genital pore is relatively closer to the anterior of the worm. The remaining 9 species described in the literature are from South America. There are no detailed analyses of larval hooklets or hamulus blades, with one exception, *P.cuvieri* (see Vaucher, 1990). Gross hamulus morphology appears to be very variable between species, for example *P.diptychi* has massive hamuli approximately 1mm in length and *P.andinum* has irregular and jagged hamuli. This is also observed within species, for example, *P.borellii* possesses notched to unnotched hamuli.

A number of authors have suggested a high degree of species-specificity for anuran polystomes (Bourgat, 1977; Bourgat & Salami-Cadoux, 1976; Combes & Channing, 1978-1979; Murith, 1981). However, Murith (1981) recorded *Polystoma gabonensis* in 3 species of *Hylarana* and found that *Ptychadena mascareniensis* harbours 3 *Polystoma* species. In addition, Vaucher (1990) reported *P.cuvieri* from 2 host species. This study has added a fourth host species for *P.nearcticum* in the United States. Moreover, anuran polystomatids may occur syntopically, for example *P.aeschlimanni* and *P.lamottei* in Togo (Bourgat &

Murith, 1980) and DuPreez & Kok (1992) discovered new species of *Polystoma* and *Metapolystoma* within the same host. Both groups of researchers concluded that syntopy was due to a change of host geographical range, possibly because of climatic change and not sympatric speciation. Experimental evidence for specificity is sparse, with only the work of Combes (1966, 1968) on European *Polystoma* and the unpublished cross-infection experiments of Bourgat & Salami-Cadoux (1976) and DuPreez & Kok (1993) to support this hypothesis. Conversely, Kok & DuPreez (1987) successfully established heterospecific infections which reached neotenic maturity and produced eggs, although at a reduced rate to worms on conspecific hosts. Successful migration and establishment of pre-destined bladder worms was also recorded. Furthermore, Kennedy (1975) and Murith (1982) suggested that although strict-specificity may exist, it may not be evident in parasite larval stages. It is evident that there are a number of exceptions to the strict host specificity suggested by Bourgat (1977), Bourgat & Salami-Cadoux (1976) Combes & Channing (1978-1979) and Murith (1981). These taxonomic problems return to the basic tenet regarding both host and parasite, that is species definition.

In conclusion, neither morphological analyses nor preliminary cross-infections separate *Polystoma* from *Hyla chrysoscelis* and *H.versicolor*. This suggests that *Polystoma nearcticum* is capable of infecting both host species and, in areas of sympatry, does so at comparable levels. This monogenean is present in 4 of the 5 documented lineages of the diploid-tetraploid complex, indicating its ancient association with its host. Furthermore, this study indicates that there is another exception to the theory that this parasite group is typically species-specific.

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Site	Host	Prevalence	Mean intensity	n
1	*H.versicolor*	26.1	1.4	46
2	*H.chrysoscelis*	29.4	1.2	17
2	*H.versicolor*	28.6	1.8	14
4	*H.chrysoscelis*	25.0	1.0	4
6	*H.chrysoscelis*	33.0	4.9	27
7	*H.versicolor*	20.0	1.0	5
10	*H.chrysoscelis*	100.0	5.0	1

Table 6.1 Infection levels of *Polystoma nearcticum* recovered from Gray treefrogs collected at 6 sites in the U.S.A. (n = host sample size).

	A.			B.		
	Mean	S.D.	n	Mean	S.D.	n
Body length (L)	6.36	0.82	14	6.12	1.17	42
Greatest width	2.21	0.29	14	2.30	0.50	42
Width at vaginae	1.26	0.15	14	1.16	0.21	42
Haptor length (HL)	1.19	0.18	14	1.11	0.19	42
Haptor width	1.89	0.20	14	1.82	0.27	42
HL/L ratio	0.18	0.10	14	0.18	0.30	42
Oral sucker length	0.26	0.03	14	0.23	0.04	42
Oral sucker width	0.48	0.04	14	0.44	0.07	42
Pharynx length	0.30	0.03	14	0.28	0.05	39
Pharynx width	0.23	0.02	13	0.21	0.03	39
Ovary length	0.76	0.06	14	0.69	0.17	42
Ovary width	0.36	0.04	14	0.37	0.09	42
Sucker diameter	0.35	0.03	84	0.32	0.04	252
Genital spine length	30.0µm	1.3µm	5	30.2µm	2.9µm	9
Hooklet I length	21.9µm	1.5µm	12	20.4µm	1.6µm	23
Hamulus length	0.36	0.03	14	0.35	0.04	42

Table 6.2 Body dimensions of sexually mature, egg producing specimens of *Polystoma nearcticum* recovered from the urinary bladder of **A.** *Hyla chrysoscelis* and **B.** *H.versicolor*. All measurements in mm, except where indicated.

	A.				B.			
Ratio	Mean	Range	S.D.	n	Mean	Range	S.D.	n
1:2	1.21	1.03-1.29	0.06	12	1.19	1.05-1.32	0.06	39
1:3	2.05	1.76-2.37	0.18	11	2.06	1.35-2.54	0.27	39
1:4	1.97	1.71-2.31	0.16	11	2.04	1.61-3.52	0.44	39
1:5	2.64	2.00-3.10	0.36	11	2.62	1.97-4.08	0.50	39
1:6	2.23	2.05-2.63	0.19	11	2.35	2.00-2.76	0.20	39
1:7	6.14	5.33-7.00	0.55	10	6.27	4.84-7.61	0.77	30
1:8	1.49	1.40-1.56	0.05	11	1.41	1.22-1.62	0.09	38
2:3	1.71	1.47-1.97	0.19	11	1.72	1.29-2.17	0.22	39
2:4	1.63	1.40-1.92	0.15	11	1.72	1.35-3.36	0.43	39
2:5	2.19	1.71-2.68	0.30	11	2.19	1.72-3.46	0.44	39
2:6	1.85	1.59-2.23	0.19	11	1.97	1.58-2.47	0.23	39
2:7	4.98	4.19-5.94	0.50	10	5.25	4 15-6.50	0.60	30
2:8	1.22	1.19-1.40	0.06	11	1.18	1.00-1.26	0.04	38
3:4	0.97	0.72-1.31	0.16	11	1.05	0 63-2.60	0.43	39
3:5	1.31	0.91-1.70	0.28	11	1.33	0.80-2.67	0.48	39
3:6	1.09	0.93-1.19	0.08	11	1.17	0 93-1.91	0.23	39
3:7	3.03	2.67-3.53	0.25	9	3.03	2.40-3.67	0.30	30
3:8	0.73	0.61-0.81	0.07	10	0.70	0.53-0.97	0.11	38
4:5	1.34	1.09-1.47	0.11	11	1.29	0.96-1.59	0.15	39
4:6	1.14	0.90-1.46	0.16	11	1.19	0.74-1.52	0.21	39
4:7	3.15	2.48-3.95	0.52	9	3.26	1.47-4.11	0.69	30
4:8	0.75	0.62-0.85	0.06	10	0.71	0.35-0.86	0.12	38
5:6	0.86	0.67-1.19	0.16	11	0.93	0.58-1.26	0.18	39
5:7	2.33	1.76-3.17	0.46	9	2.53	1.20-3.44	0.56	30
5:8	0.56	0.48-0.64	0.06	10	0.55	0.34-0.69	0.09	38
6:7	2.75	2.23-3.29	0.29	9	2.66	1.79-3.65	0.41	30
6:8	0.67	0.53-0.76	0.06	10	0.60	0.47-0.76	0.08	38
7:8	0.24	0.20-0.29	0.03	10	0.23	0.18-0.29	0.03	30

Table 6.3 Ratios of hamulus morphology for **A.** *Hyla chrysoscelis* and **B.** *H.versicolor* derived *P.nearcticum*. Details of measurements given in the Methods.

	Posterior sucker pr /Days p.i.	Middle sucker pr /Days p.i.	Anterior sucker pr /Days p.i.
H.versicolor -> H.versicolor	6	8	12
H.versicolor -> H.chrysoscelis	4	8	10
H.chrysoscelis -> H.chrysoscelis	6	10	12
H.chrysoscelis -> H.versicolor	6	8	10

Table 6.4 Time (days p.i.) to the first appearance of the haptoral sucker pairs for both conspecific and heterospecific infections.

6.7 Legends.

Fig.6.1 The distribution of sampling sites of *Hyla chrysoscelis* and *H.versicolor* in the U.S.A. (The sites are numbered as described in the text).

Fig.6.2 Frequency distribution of *P.nearcticum* in A) *Hyla chrysoscelis* and B) *H.versicolor* from 4 major sampling sites. (Localities indicated in the text).

Fig.6.3 *P.nearcticum*: Sexually mature, egg producing adult recovered from *H.versicolor*. **Abbreviations**: **an**, anastomose; **c**, cirrus; **gc** & **[illegible]**, gut caecum; **ham**, hamulus; **os**, oral sucker; **ov**, ovary; **ph**, pharynx; **suc**, haptoral sucker; **ut**, uterus; **vd**, vas deferens. Vitellaria not indicated. **Scale bar** = 1 mm.

Fig.6.4 Haptoral hamulus morphology from 6 different specimens of *P.nearcticum* recovered from A) *Hyla chrysoscelis* and B) *H.versicolor* at a sympatric and syntopic site in Phelps Co., Missouri. **Scale bar** = 0.1mm.

Fig.6.5 Geographical variation of *P.nearcticum* haptoral hamulus morphology from 6 different specimens recovered from *Hyla chrysoscelis in* A) Louisiana and B) Florida. **Scale bar** = 0.1mm.

Fig.6.6 Scatter diagram of total hamulus length and mean blade length for *P.nearcticum* recovered from *Hyla chrysoscelis* (+) and *H.versicolor* ([illegible]).

Fig.6.7 Scatter diagram of hooklet I length (b) and length to the bifurcation of the guard (a) for *P.nearcticum* recovered from *Hyla chrysoscelis* (+) and *H.versicolor* ([illegible]).

Fig.6.8 Total body length (T) and haptor length (H) to 14 days p.i. for A) *H.versicolor* oncomiracidia -> *H.versicolor* tadpoles; B) *H.versicolor* -> *H.chrysoscelis*; C) *H.chrysoscelis* -> *H.chrysoscelis* and D) *H.chrysoscelis* -> *H.versicolor*. (S.D. indicated, n = no. of worms).

Fig.6.9 Pharynx length to 14 days p.i. for A) *H.versicolor* oncomiracidia -> *H.versicolor* tadpoles; B) *H.versicolor* -> *H.chrysoscelis*; C) *H.chrysoscelis* -> *H.chrysoscelis* and D) *H.chrysoscelis* -> *H.versicolor*. (S.D. indicated, n = no. of worms).

Fig.6.10 Development of the posterior sucker pair to 14 days p.i. for A) *H.versicolor* oncomiracidia -> *H.versicolor* tadpoles; B) *H.versicolor* -> *H.chrysoscelis*; C) *H.chrysoscelis* -> *H.chrysoscelis* and D) *H.chrysoscelis* -> *H.versicolor*. (S.D. indicated, n = no. of worms).

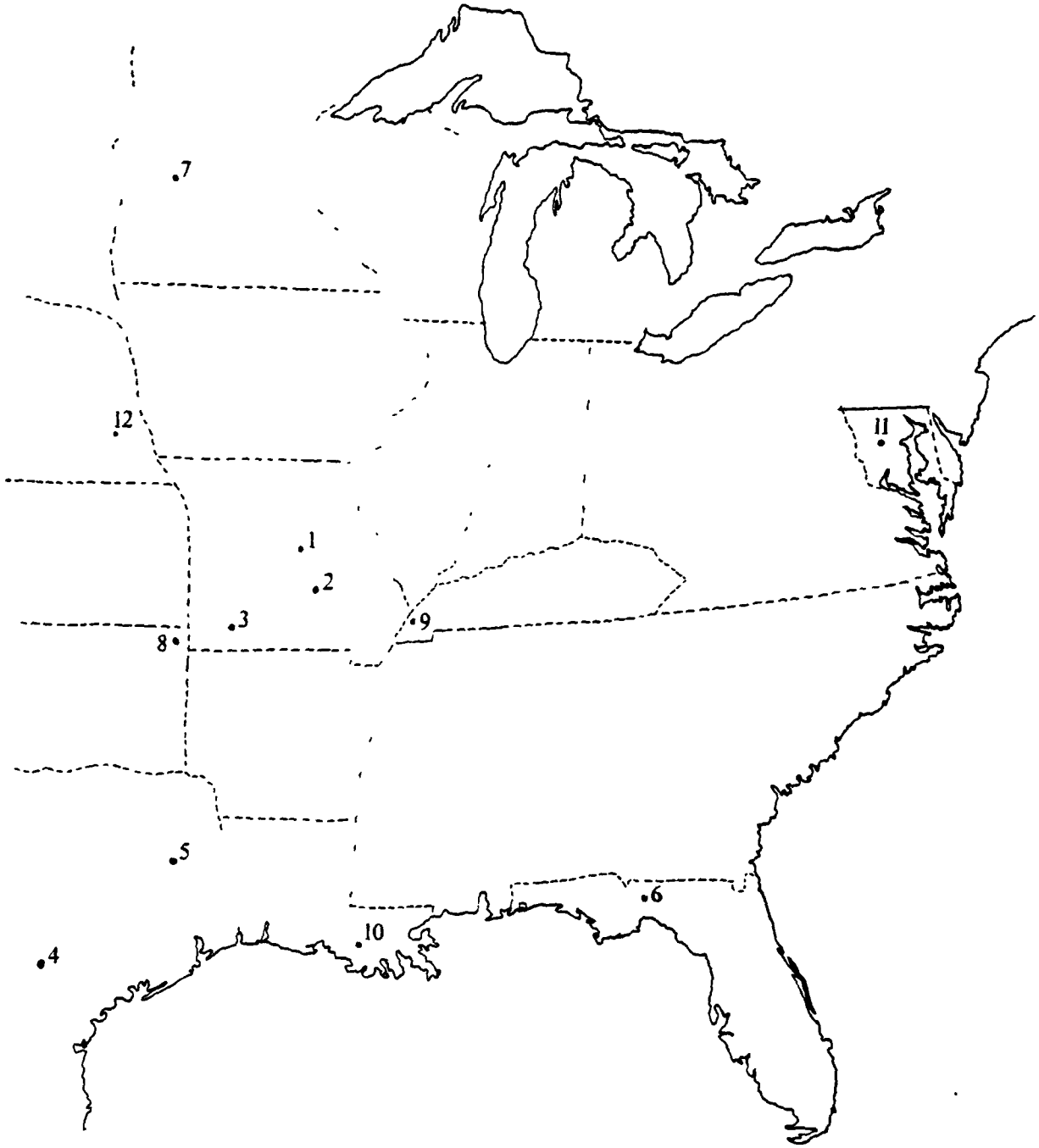

Fig.6.1

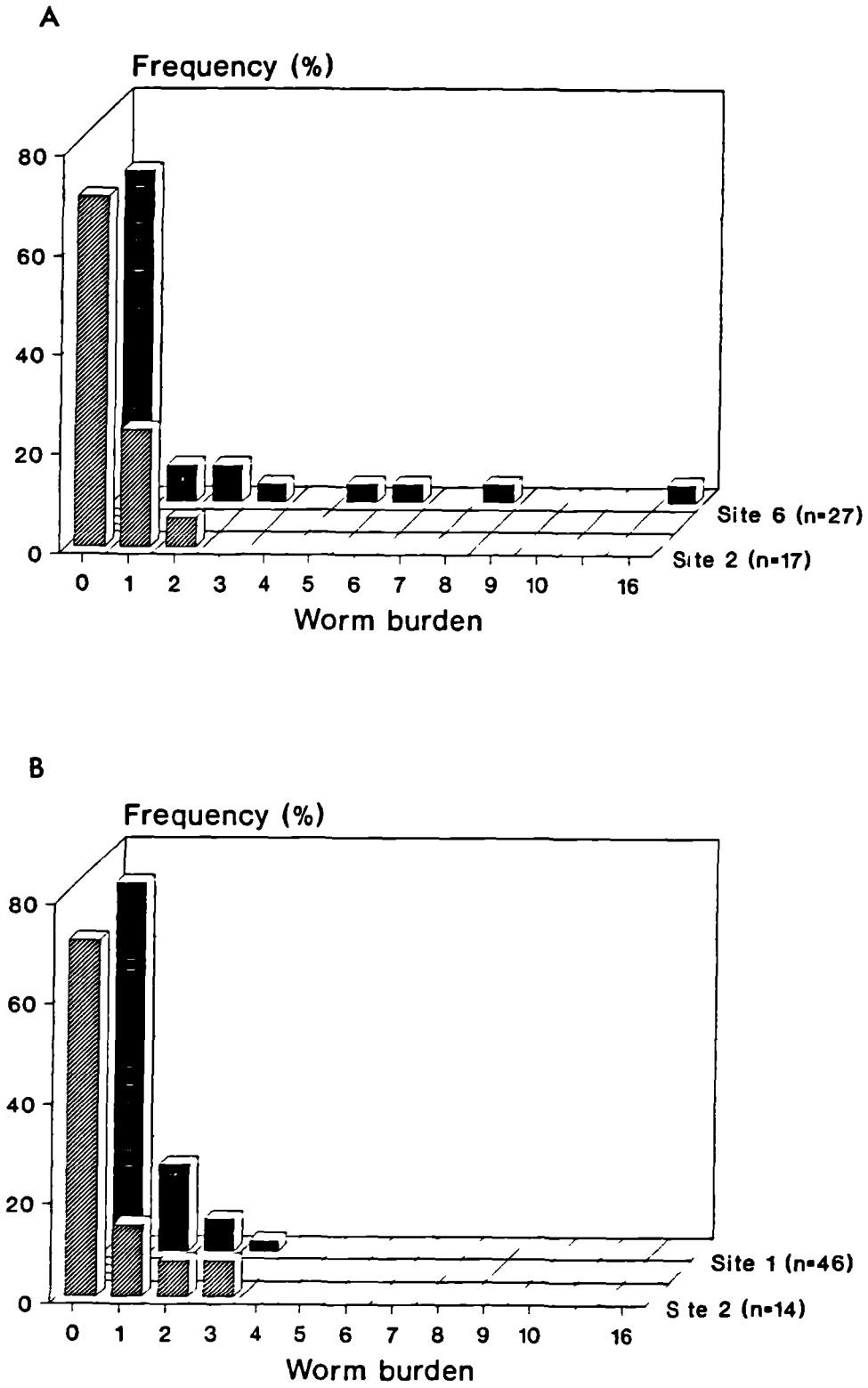
A
Frequency (%)
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 16
Worm burden
Site 6 (n=27)
Site 2 (n=17)
B
Frequency (%)
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 16
Worm burden
Site 1 (n=46)
S te 2 (n=14)

Fig.6.2

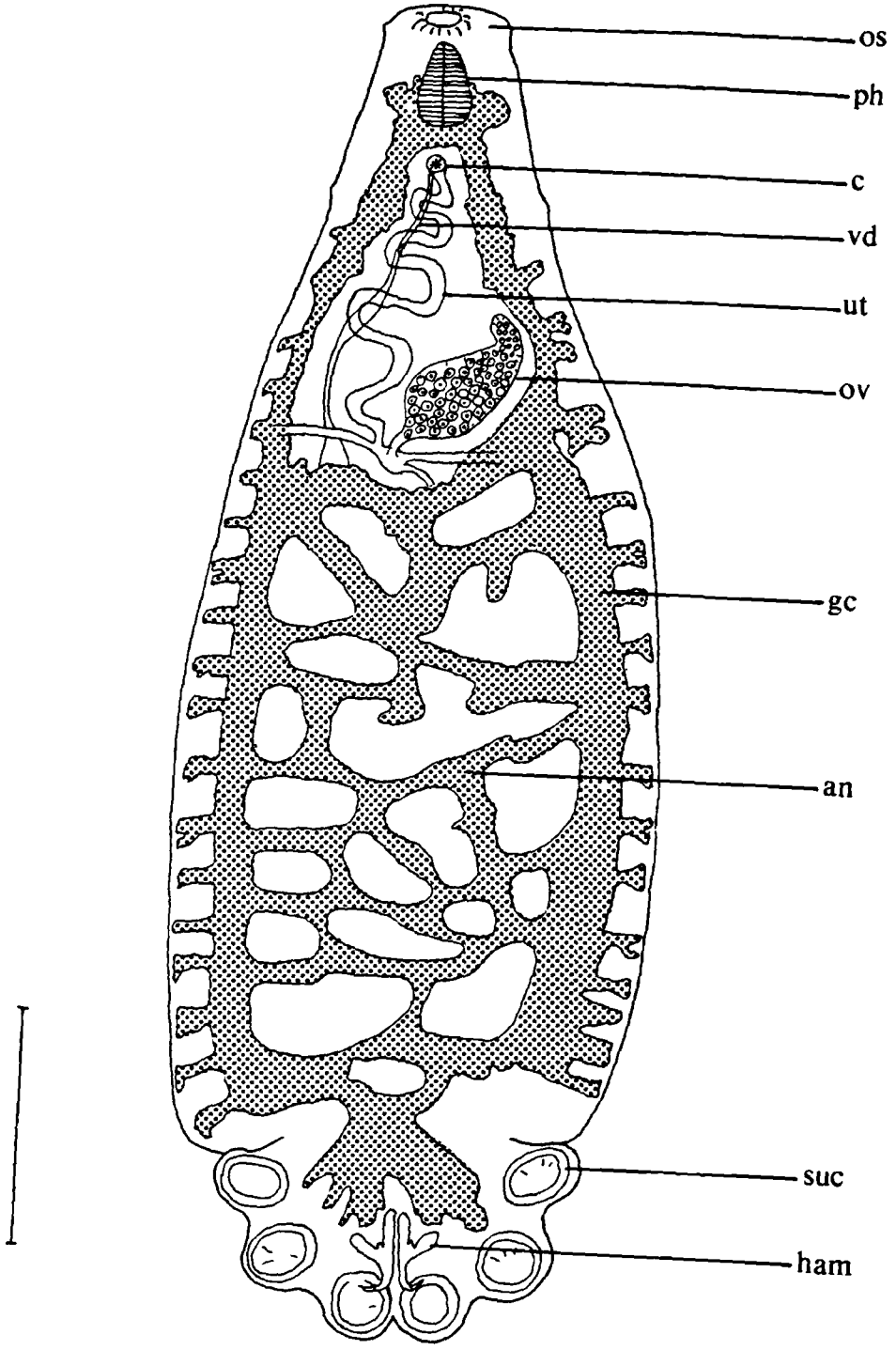

Fig.6.3

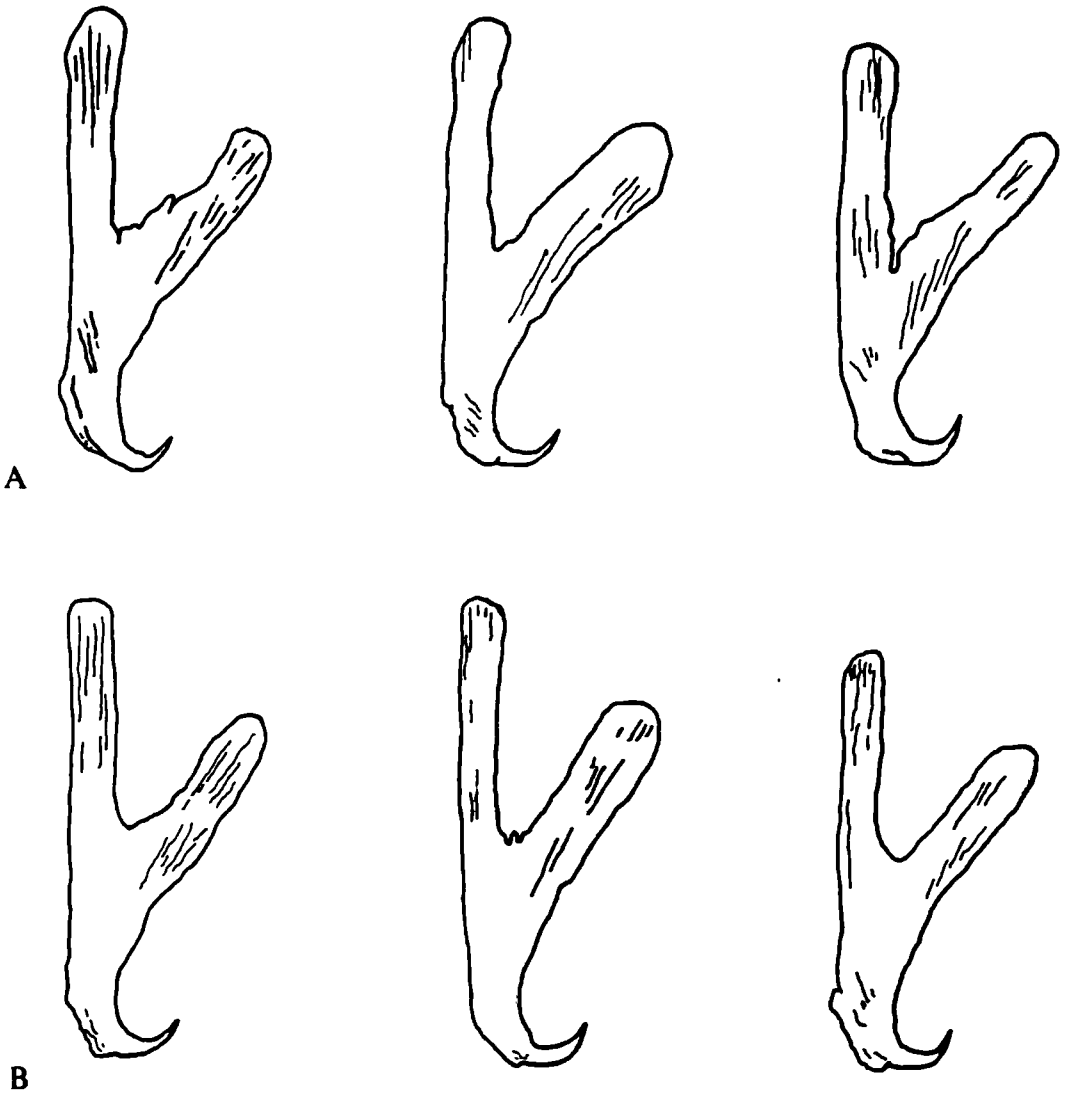

Fig.6.4

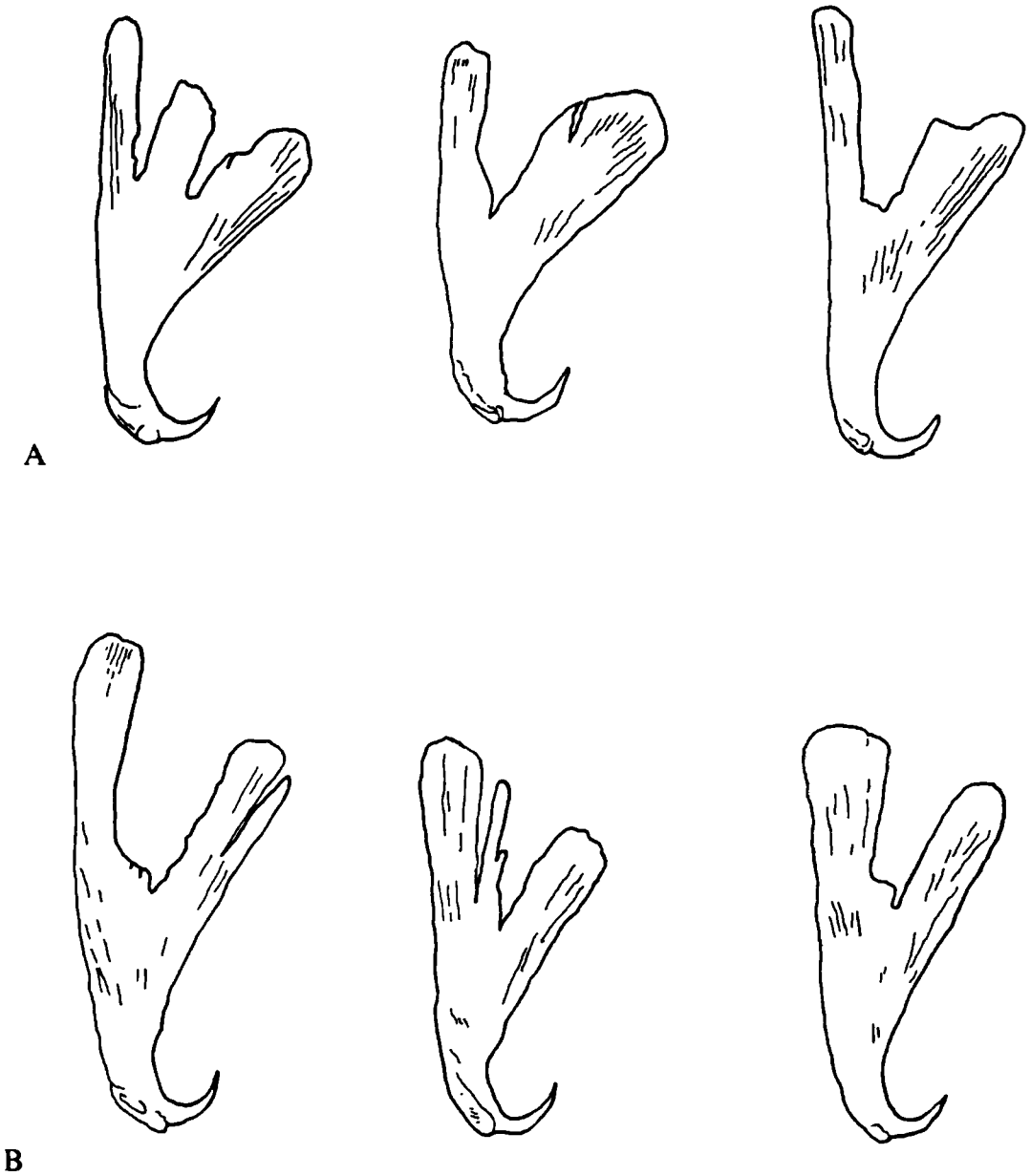

Fig.6.5

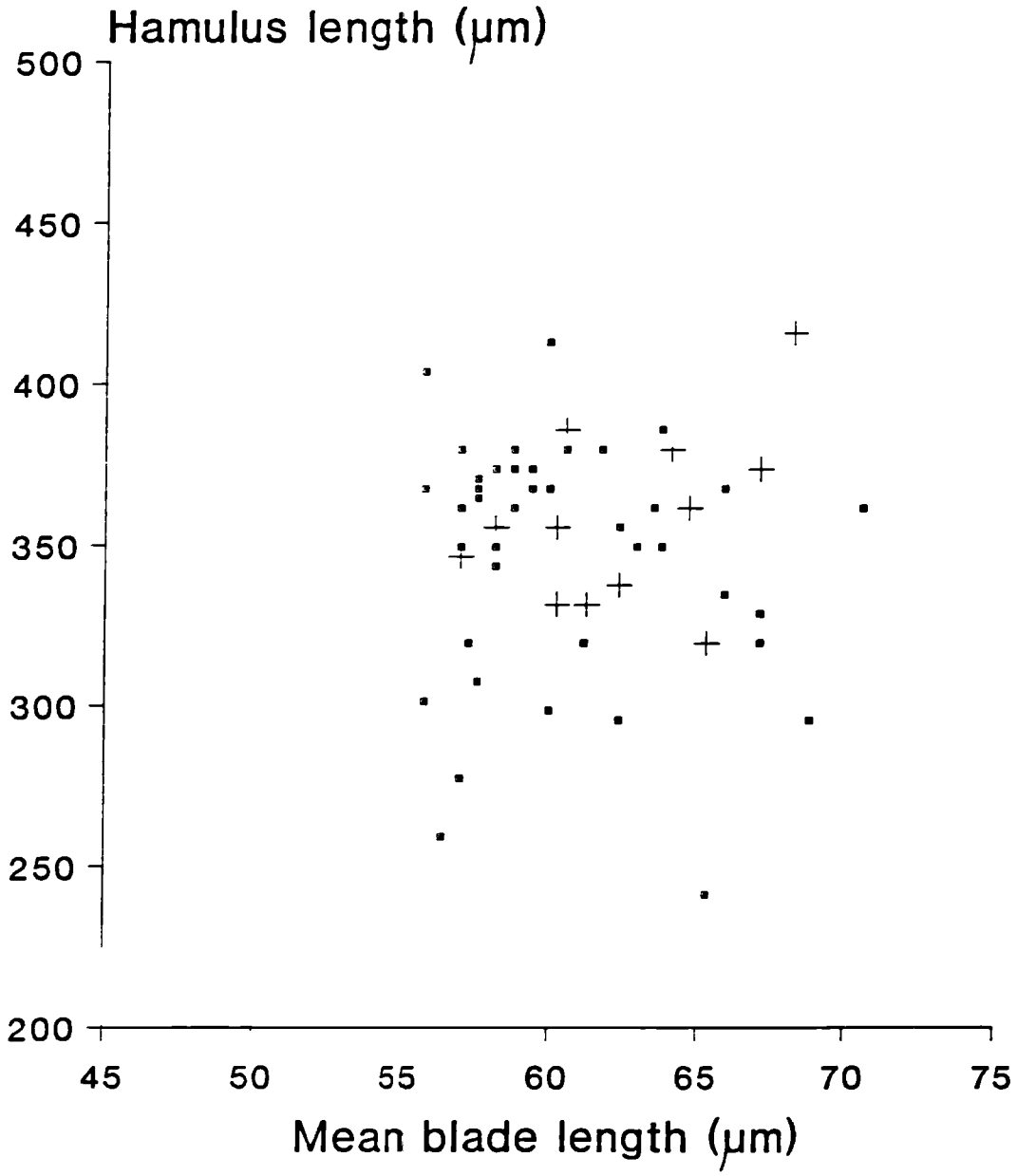
Hamulus length (µm)
500
450
400
350
300
250
200
45
50
55
60
65
70
75
Mean blade length (µm)

Fig.6.6

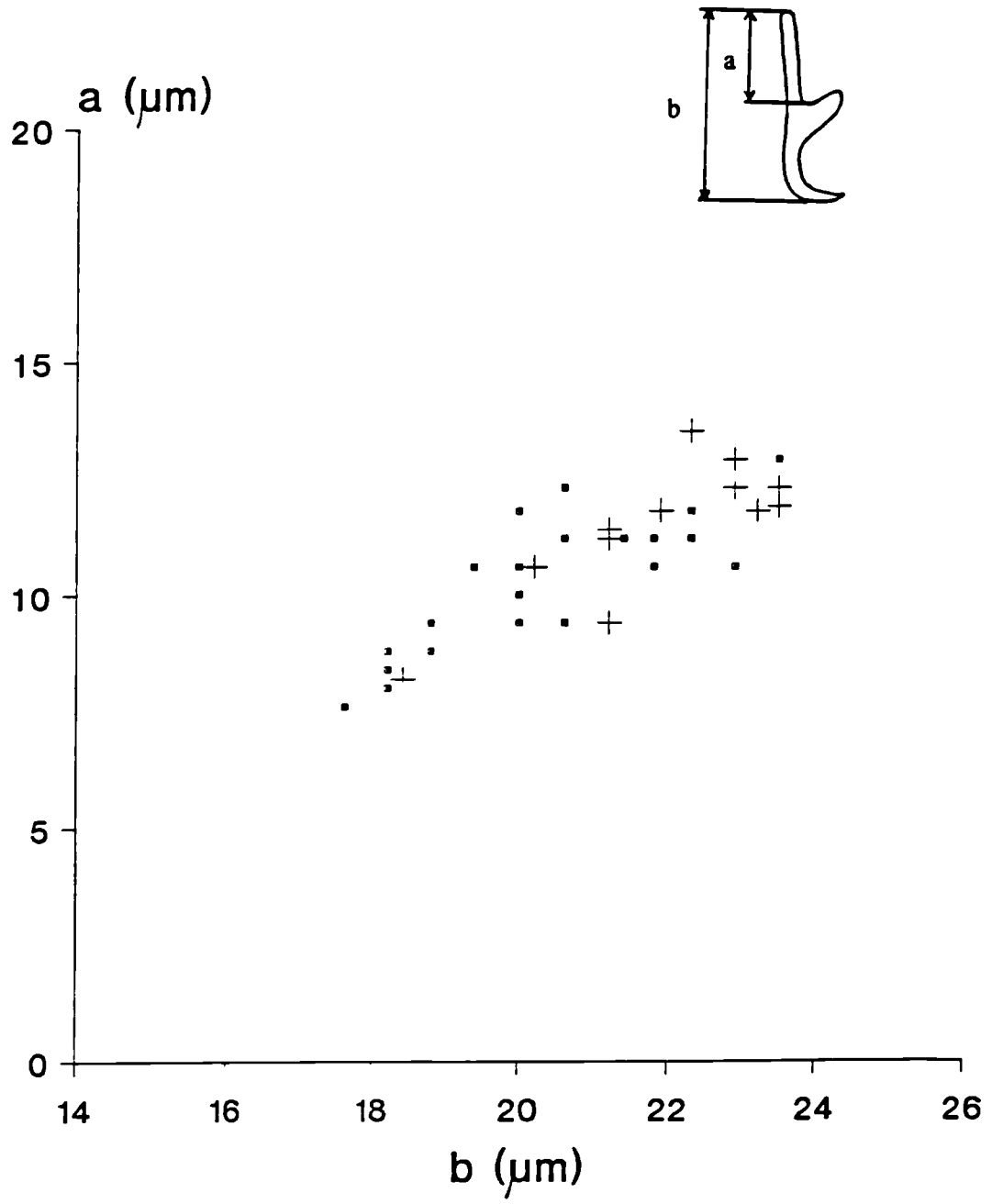

Fig.6.7

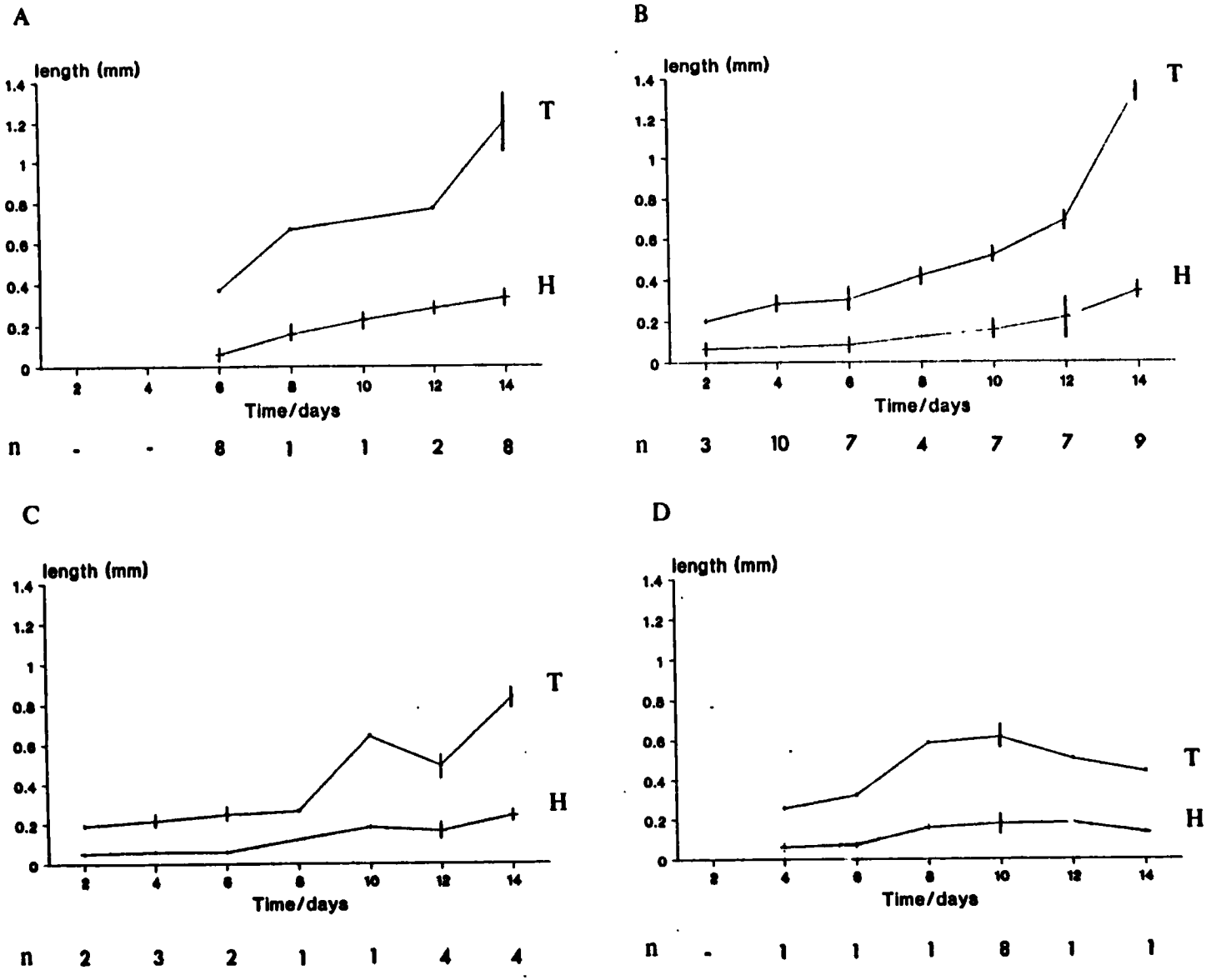
A
length (mm)
1.4
1.2
1
0.8
0.6
0.4
0.2
0
T
H
2
4
6
8
10
12
14
Time/days
n - - 8 1 1 2 8
B
length (mm)
T
H
Time/days
n 3 10 7 4 7 7 9
C
length (mm)
T
H
Time/days
n 2 3 2 1 1 4 4
D
length (mm)
T
H
Time/days
n - 1 1 1 8 1 1

Fig.6.8

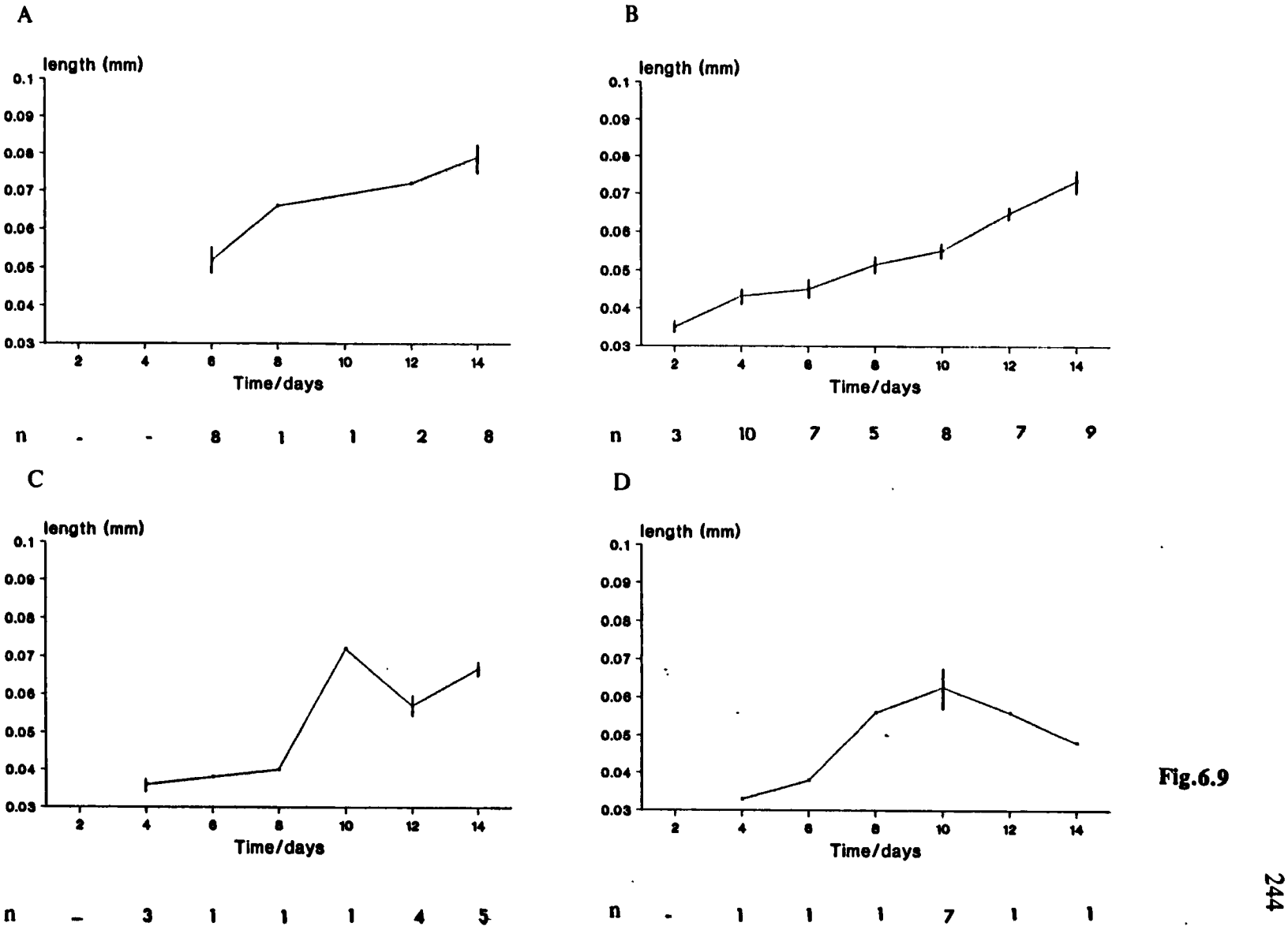
A
length (mm)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
2 4 6 8 10 12 14
Time/days
n - - 8 1 1 2 8
B
length (mm)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
2 4 6 8 10 12 14
Time/days
n 3 10 7 5 8 7 9
C
length (mm)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
2 4 6 8 10 12 14
Time/days
n - 3 1 1 1 4 5
D
length (mm)
0.1
0.09
0.08
0.07
0.06
0.05
0.04
0.03
2 4 6 8 10 12 14
Time/days
n - 1 1 1 7 1 1

Fig.6.9

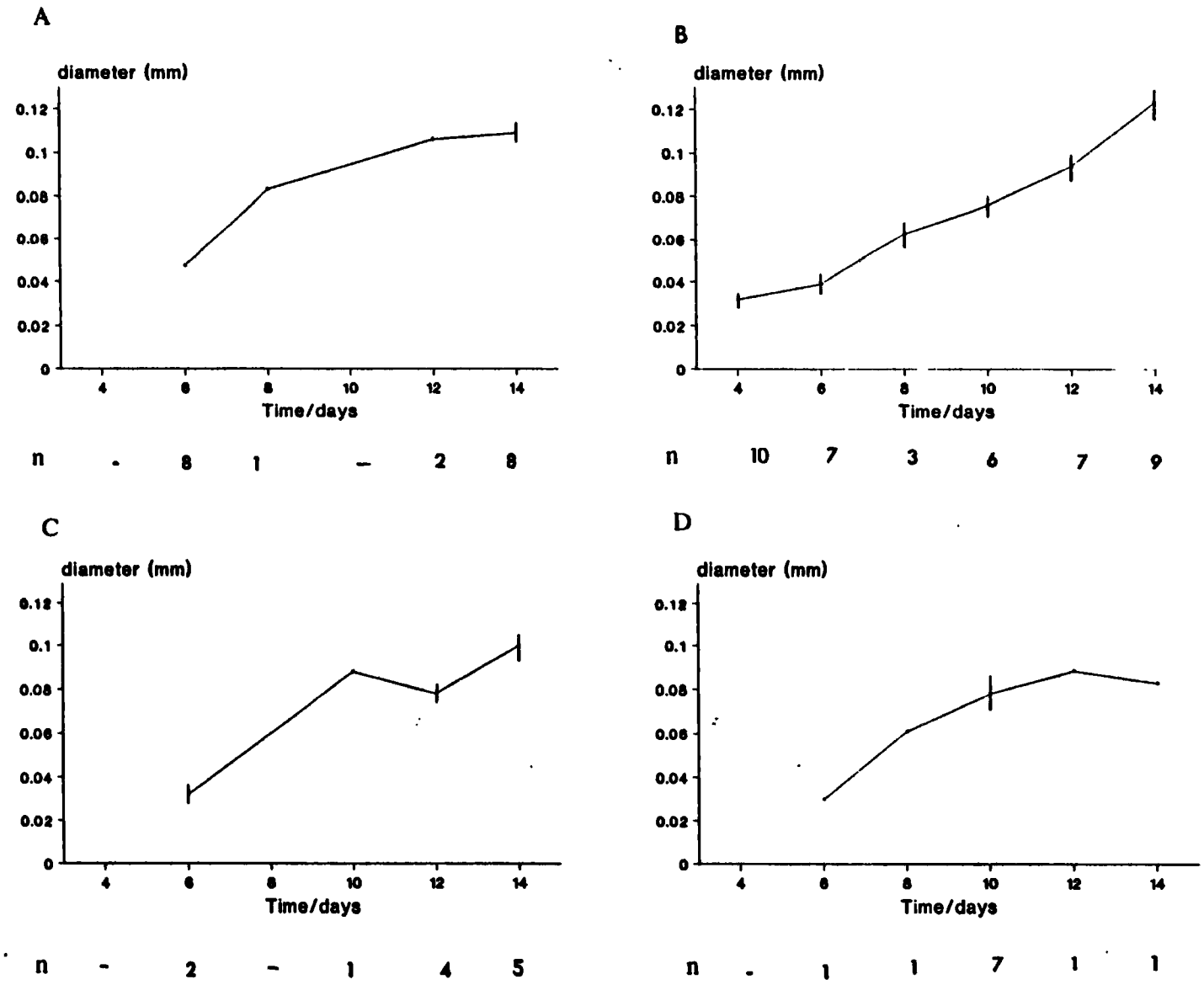
A
diameter (mm)
0.12
0.1
0.08
0.06
0.04
0.02
0
4
6
8
10
12
14
Time/days
n - 8 1 - 2 8
B
diameter (mm)
0.12
0.1
0.08
0.06
0.04
0.02
0
4
6
8
10
12
14
Time/days
n 10 7 3 6 7 9
C
diameter (mm)
0.12
0.1
0.08
0.06
0.04
0.02
0
4
6
8
10
12
14
Time/days
n - 2 - 1 4 5
D
diameter (mm)
0.12
0.1
0.08
0.06
0.04
0.02
0
4
6
8
10
12
14
Time/days
n - 1 1 7 1 1

Fig.6.10

Chapter 7.

The establishment, developmental rate and reproduction of neotenic *Polystoma nearcticum* (Paul, 1935) Price, 1939 (Monogenea:Polystomatidae) in experimentally infected tadpoles of *Hyla versicolor* (Anura:Hylidae).

7.1 Abstract.

The establishment, development, life-cycle strategy and reproduction of neotenic *Polystoma nearcticum* was investigated in experimentally infected *Hyla versicolor* tadpoles, their natural anuran host. Oncomiracidia rapidly entered the single spiracle on the right side of the body. Upon establishment, parasites attached to gill filaments on the open side of the branchial chamber. Initial distribution of worms at 2 days post-infection (p.i.) was 94.9% recovered from the right gill with the remaining 5.1% on the left gill. This preference was maintained over the experimental period. The overall developmental rate of *P.nearcticum* was recorded over 30 days at constant temperature (20.6+_0.8°C) and the differential development of the haptoral sucker pairs provides a means of age determination up to 20 days p.i.

At 10 days p.i. no morphological differences were recorded between worms infecting tadpoles less than 10 days post hatch (p.h.) and those at 24 days p.h. This is discussed in relation to the neotenic and 'bladder-destined' courses of the life-cycle and the known developmental rate of the host.

Sexual maturity of neotenics was reached between 18 and 22 days p.i. and egg production was monitored to 30 days p.i. The production of eggs followed a typical pattern for all infrapopulation densities, with a gradual increase in output over 2-3 days until a steady rate was achieved. Regression analysis revealed that neotenics were significantly smaller at higher burdens (R^2 = 30.5%, F = 7.89, p = 0.012) and when host weight/parasite was low (R^2 = 54.8%, F = 21.80, p <

0.001). Smaller worms produced fewer eggs (R^2 = 40.9%, F = 12.48, p = 0.002) contributing to a density-dependent effect for egg production/parasite in burdens of 3 to 9 worms/host (R^2 = 47.7%, F = 11.87, p = 0.004).

7.2 Introduction.

Polystoma nearcticum was first described by Paul (1938) from the Green treefrog, *Hyla cinerea* and the tetraploid Gray treefrog, *H.versicolor* in the United States. As described in Chapter 6, the diploid Gray treefrog, *H.chrysoscelis* has also been recorded as a host for this monogenean. There have been no further experimental investigations of *P.nearcticum*, although the Gray treefrogs have been extensively studied for their behaviour (Gayou, 1984; Gerhardt, 1974, 1978; Hausfater, Gerhardt & Klump, 1990; Klump & Gerhardt, 1987; Morris, 1989), ecology (Ritke & Babb, 1991; Ritke, Babb & Ritke, 1990, 1991), neurophysiology (Gerhardt & Doherty, 1988), morphology (Matson, 1990a, b), evolutionary biology and genetics (Danzmann & Bogart, 1981, 1982; Maxson, Pepper & Maxson, 1977; Ralin & Selander, 1979; Wiley, 1983).

Paul (1938) recorded a life-cycle closely related to that of the European *Polystoma integerrimum* (Gallien, 1932a, 1932b, 1934, 1935). *P.nearcticum* exhibits the same dimorphic development as *P.integerrimum*, with one path leading to rapid sexual maturity, the 'neotenic' stage, which does not survive host metamorphosis. The role of the neotenic is to produce a steady 'trickle' infection, to augment the punctuated deposition of adult-derived eggs on host breeding nights. Infrapopulation densities of neotenics may decline during host development

as Kok (1990) documented high mortality rates for the branchial form of *P.umthakathi*.

Alternatively, juvenile worms may take the 'normal' developmental path, these are the 'bladder-destined' worms which exhibit a much slower developmental rate, moving from the gills during host metamorphosis, to the definitive site, the urinary bladder. It was originally believed that migration was via the alimentary tract (Gallien, 1932b). However, Combes (1967) discovered that the worms made a rapid movement over the ventral surface at night. The parasite then reaches sexual maturity in close synchronisation with its host (Miretski, 1951).

The reproductive strategy of *P.nearcticum* must be closely linked to the complex behaviour of the host. The deposition of eggs from adult worms is limited to periods when the host enters water, for example on breeding nights. This restricted window for transmission occurs when temperature and humidity rise above threshold levels during the two month breeding season (Hausfater *et al.*, 1990).

Male *Hyla chrysoscelis* call on average for 1.47 to 2.56 nights each breeding season. Once paired, the frogs may remain in amplexus for up to 5 hours but actually enter the water for a much shorter period (Morris, 1989). With the brief opportunity for adult-derived eggs to be released into the correct environment, the neotenic cycle will provide an alternative route for infection.

In the New World, a number of anuran polystomatids have been recorded from Central and South America (Caballero & Cerecero, 1941; Combes & Laurent, 1974, 1978, 1979; Vaucher, 1981, 1986, 1987, 1990; Vigueras, 1955) but little of their biology is known. However, in the Old World, anuran polystomatids have been the subject of detailed research (Combes, 1967, 1968; DuPreez & Kok, 1992, 1993; Jackson & Tinsley, 1988a, b; Kok, 1990; Kok & DuPreez, 1987, 1989; Kok & Seaman, 1987, Kok & van Wyk, 1986; Maeder, 1973; Maeder, Euzet & Combes, 1970; Murith, 1981a, b, 1982; Murith, Mirmad-Gassman & Vaucher, 1978; Tinsley, 1983; Tinsley & Owen, 1975). Neotenic forms have been recorded from just under half of the *Polystoma* species recovered from Africa, *P.africanum, P.australis, P.baeri, P.dorsalis, P.ebriensis, P.gabonensis, P.gallieni, P.grassei, P.mangenoti, P.marmorati, P.mashoni, P.perreti, P.togoensis and P.umthakathi* (see Van Niekerk, 1992). The neotenic biology of *P.australis* and *P.umthakathi* has been examined in detail by Kok & DuPreez (1987, 1989) and Kok (1990).

The remits of this study were to document the establishment and distribution of branchial *P.nearcticum*. A rate of morphological development was to be established, with sexual maturity and rates of egg production assessed for single and multiple burdens. The change from the neotenic to 'bladder-destined' form in the life-cycle is dependent on the age of the host tadpole when first encountered (Gallien, 1934; Murith, 1981a). Murith (1981a) found that the age of the tadpoles at which the change occurred was imprecise and it was possible to obtain a mixture of the two forms on the same tadpole from the same infection event.

Ritke, Babb & Ritke (1990) recorded that in Tennessee, *H.chrysoscelis* may reach metamorphosis in 35 days and Paul (1938) stated that neotenic *P.nearcticum* first produced eggs 22 days after infection, therefore neotenics will have a brief period in which to supplement infection levels prior to host metamorphosis. It was hoped to infect tadpoles of various ages under standard conditions to document the changeover period for *P.nearcticum.*

7.3 Materials and Methods.

Fieldwork was based at two large ponds within the Ashlands Reserve, 35 km south of Columbia, Missouri. Twelve egg masses were collected from amplexed *H.versicolor* on the nights of 15 and 16th May 1992 and returned to the laboratory. Each clutch was examined under a binocular microscope for *P.nearcticum* eggs but all parents were uninfected. Individual clutches were maintained in aquaria (approx. 30x15x20cm) and once all the eggs had hatched, tadpoles were transferred by pipette into paddling pools (approx. 1m diameter) filled to a depth of 10cm. Both the aquaria and pools were filled with aged water and aerated.

After 10 days the tadpoles were fed daily on boiled green leaf lettuce. The water was also changed each day after hand-netting the tadpoles into an aquarium. Water temperature was maintained at 20.6+_ 0.8^{0}C with thermostatically controlled air-conditioning units. The temperature was monitored at 9.00am, 12.00, 15.00 and 20.00 by a Jencon electronic thermometer.

In the field, male *H.versicolor* were readily located by their loud trill call and were captured by hand. Their calling sites were typically close to the water in the lower branches of trees, shrubs and undergrowth. Females do not call and so unless already close to a calling male or in amplexus they were only encountered by chance. Each frog was either put singly into a small box or in an aquarium with up to 15 frogs in total. Both types of receptacle were filled to a depth of approximately 2cm with aged water.

Upon return to the laboratory, the water was collected into crystallising dishes, plus 2-3 washings of the container. The eggs were allowed to settle before excess water was decanted. The eggs were concentrated into the centre by swirling the final volume of water in the dish. They were drawn into a pasteur pipette and released into small (5cm diameter) petri dishes which were maintained at the same temperature as the tadpoles. Oncomiracidia typically hatched 9-11 days after oviposition.

Single tadpoles were exposed to 10 oncomiracidia each in 5cm diameter petri dishes containing 15ml of aged water. Recently hatched oncomiracidia (less than 1h old) were gently taken up into a pasteur pipette and released in the centre of the infection dish with the minimum current. All oncomiracidia were counted under a binocular microscope as they entered the infection dish. The infection process was observed using a binocular microscope during a number of exposures of tadpoles to oncomiracidia. After 24h the tadpoles were moved to larger containers (plastic drinking cups) for easier care.

Different maintenance and dissection timings were required for each separate experiment:

7.3.1 Establishment and developmental rate.

300 tadpoles aged 7-14 days post hatch (p.h.) were individually exposed to oncomiracidia as described in the above regime and then a sub-sample of 10 individuals was dissected at 2 day intervals from 2 to 30 days p.i.

7.3.2 The timing of the changeover from the neotenic to the 'bladder-destined' form.

Tadpoles were infected as above with the only variable being the age of the host. 10 tadpoles at each age (4, 6, 8, 10, 16, 18 & 24 days p.h.) were individually exposed to oncomiracida, depending upon availability, and dissected after 10 days.

7.3.3 Neotenic egg production.

A sub-sample of 35 tadpoles from Section 7.3.1 was placed in cups with a mesh insert after 15 days. The insert sat inside the original cup and was formed by cutting a second cup two thirds along its length and replacing the bottom with a fine mesh. This allowed any eggs released to pass through to the bottom, preventing ingestion by the host. Egg production was monitored every 24h and tadpoles were dissected after 3 comparable totals were recorded, indicating that the neotenics were at a stable rate of production. As the burden on each tadpole was not known until dissection this assessment could only be arbitrary.

Dissection of tadpoles was undertaken as described in Chapter 6. In the early stages of development, pigment does not obscure the gill chamber of the tadpole and an estimate of the intensity of infection could be made prior to dissection. However, invariably not all the worms could be observed in this way, particularly those at the margins of the gill close to the spiracle. This was the first section of the branchial chamber to become obscured by pigment in older tadpoles.

The sites of attachment were recorded and all worms were fixed under standard (18x18mm) coverslip pressure in a 10% formal saline solution. On return to Q.M.W. the worms were stained and mounted *in toto* for morphological analysis as described in Chapter 3. Measurements of length and width were taken for the body, haptor, pharynx, ovary, genital spines, eggs, oral and haptoral suckers using a Nikon Optiphot UFX IIA microscope. Camera lucida drawings were also made of representatives of each age class of neotenic *P.nearcticum* using the same microscope.

7.4 Results.

7.4.1 Establishment and developmental rate.

Although there was some variation, the eggs typically hatched *en masse* between 17.00 and 21.00 h 9-11 days after oviposition. The life-span of *P.nearcticum* larvae may approach 24 h, by which time their motion appeared laboured with repeated swimming in tight circles prior to death.

The infection process followed a characteristic pattern with the larvae attaching to the body of the tadpole usually after the first close encounter. The point of initial attachment varied, however, in all cases migration to the branchial chamber via the single spiracle was rapid taking only a few minutes. The juvenile worms become hidden within the filaments until their guts become pigmented following the first blood meal.

After 48 h tadpoles exposed to infective stages under the above regime had a 100% prevalence with a mean intensity of 4.9 worms (n = 12). Of the 59 worms recovered from these 12 tadpoles all had fed on blood with 56 (94.9%) recovered from the right gill and only 3 (5.1%) on the left. As shown by Fig.7.1, after establishment the vast majority of worms remained on the right side of the chamber. This trend continued even when the area became crowded in the heaviest burdens. As the tadpoles and worms developed, a number of neotenics transferred their haptors from the gill filaments to the emerging front limb buds in the branchial chamber. This was first noted at 22 days p.i. and only on the right side.

As noted in the Introduction, the invading worms may take one of two life-cycle courses depending upon the age and physiology of the host tadpoles, only neotenic forms are considered for the following descriptions.

7.4.1.1 Developmental rate of neotenic *Polystoma nearcticum*.

Tadpoles infected with *P.nearcticum* under standard conditions were dissected at 2 day intervals from 2 to 30 days p.i. and neotenic worms recovered from the branchial chamber.

Morphological development.

A suite of measurements was recorded to document the morphological development of *P.nearcticum*. As worm burdens ranged from 1-10 parasites/host and burden size has a significant effect on mean parasite body length (see Section 7.4.3 below) only burdens of 1-5 were considered for Figs.7.2, 7.3, 7.4 & 7.5. There was a high degree of variation at all burdens, however, the total body and haptor length, haptoral sucker diameter and pharynx dimensions were the most consistent measure of developmental rate with the lowest coefficient of variation.

Total body length and pharynx dimensions.

Total body length increased, with some degree of variation until 20 days p.i. at which point the rate slowed (Fig.7.3), coinciding with neotenic maturity. Furthermore, there is perceptible increase in the growth rate of total body length between 12 and 20 days p.i. Pharyngeal length increased in a similar manner to body and haptor length (Fig.7.4). However, there are marked drops in the rate of

growth for all measurements at 16 and 26 days p.i. (Figs. 7.3 & 7.4), which is discussed below in relation to sample size, host weight and fixation.

Haptoral dimensions.

The length of the haptor increased, but with less variation than the overall body length and with a less pronounced increase during 12-20 days p.i. (Fig.7.3). The oncomiracidium bears 16 marginal hooklets (nomenclature following Llewellyn, 1963) but no suckers on the muscular haptor. It is these hooklets that provide the initial attachment for the parasite (their dimensions are described below). The suckers formed over the first 12 days after establishment. There was a differential development of the three pairs of suckers (posterior, middle and anterior).

The posterior pair became apparent after 6 days p.i. and are clearly defined by 8 days p.i. (Fig.7.2A). The middle pair were beginning to form in 1/9 specimens fixed at 6 days p.i., however, at 8 days p.i. their outline was formed in all specimens (Fig.7.2A). The anterior pair were beginning to enclose hooklets V at 8 days p.i. and all suckers are fully differentiated by 12 days p.i. The difference in diameter of the pairs of suckers reduced over time and were close to parity by 30 days p.i. (Fig.7.5). Therefore, the suckers are a useful measure of age up to neotenic maturity. The hooklets were readily distinguishable in the very earliest stages of development but become obscured in more mature specimens.

Intestinal arrangement.

Up to 10 days p.i., the gut consisted of two simple caeca branching immediately posterior to the pharynx and rejoining in the haptor (Fig.7.2B). By 14 days p.i., a number of diverticula had formed and anastomoses were common, beginning posterior to the developing ovary through to the haptor (Fig.7.2C).

Reproductive system.

The ovary (distinguished as a darkly staining region posterior to the pharynx) is apparent after 6 days p.i., this ovoid body (8 days p.i., Fig.7.2A) slowly changed shape to an elongate, almost rectangular form by 14 days p.i. (Fig.7.2C). In the first 12 days of development the ovary often became obscured by the gut in fixed specimens. After this time the full reproductive system become clear, the ducts and canals were pronounced at 16 days p.i. (Fig.7.2D) and eggs were formed in the ootype at 18 days p.i. (Fig.7.2E).

7.4.1.2 Description of the mature neotenic form of *Polystoma nearcticum*.

The description of the mature neotenic form is based on the morphological analyses of twenty gravid parasites (30 day p.i.) recovered from experimentally infected tadpoles at burdens of 4 (n = 14) and 6 (n = 6). Measurements are in mm (except where indicated) with the range, mean and standard deviation recorded. A summary of the main characters is recorded in Table 7.1.

The overall body form (Fig.7.6) is comparable to the adult described in Chapter 6, the anterior region is lanceolate in shape with the haptor at the posterior. The

parasite measures 1.50-3.87 (2.81 +_ 0.58) in total body length with a maximum width of 0.48-1.06 (0.79 +_ 0.16). The haptor ranges from 0.36 to 0.72 (0.58 +_ 0.09) in length from 0.64 to 1.36 (1.05 +_ 0.19) in width. The overall haptor length to total body length ratio is 0.207.

Haptor.

The haptor bears three pairs of muscular suckers with hamulus primordia situated between the first pair of suckers. The marginal hooklets were 17.6-23.5μm in length, comparable to 2 day p.i. specimens. As noted above the suckers developed at slightly different rates with the posterior (1) pair measuring 0.12-0.18 (0.16 +_ 0.01), middle (2) pair measuring 0.11-0.17 (0.15 +_ 0.02), anterior (3) pair measuring 0.10-0.16 (0.14 +_ 0.02) at 30 days p.i.

Intestinal arrangement.

The pyriform muscular pharynx lies just behind the mouth at the anterior of the body. The pharynx length measures 0.08-0.14 (0.12 +_ 0.01) in length and in width from 0.09 to 0.13 (0.11 +_ 0.01). The intestine bifurcates just behind the pharynx, forming two caeca which run longitudinally along the body margins rejoining in the anterior region of the haptor. There are numerous branched and unbranched lateral and median diverticula. Anastomoses are formed posterior to the reproductive system and reach into the haptor. There are numerous diverticula which extend into the haptor.

Reproductive system.

The overall structure of the reproductive system is shown in Fig.7.7. The ovary is in the anterior third of the body measuring 0.38-0.73 (0.61 +_ 0.09) in length and 0.10-0.22 (0.15 +_ 0.03) in width and is rectangular in shape. The testis is spherical lying posterior to the ovary and is often hidden by other organ systems. The genital pore bears 8 spines with a mean length of 16μm (+_0.5μm). Vitelline collecting ducts feed into the ootype from both sides of the body. Vaginae are absent, and eggs are formed in the ootype immediately prior to release, a uterus being absent. The eggs measured 0.300 (+_0.02) in length and 0.150 (+_0.001) in width. No genito-intestinal canal was observed.

7.4.2 The timing of the changeover from the neotenic to the bladder form.

It proved difficult to obtain oncomiracidia hatching at 2 day intervals as fieldwork egg collections were irregular. Furthermore, neotenic-derived eggs hatched at the end of the study period, leaving insufficient time for exposures under this regime. An attempt was made to delay the hatching of sub-samples of adult-derived eggs by moving them from the natural photoperiod to continuous darkness 8 days after collection. Their return to the natural photoperiod was then staggered. Ultimately, it was only possible to expose tadpoles to infective stages at 4, 6, 8, 10, 16, 18 and 24 days p.h. Worms were recovered from 4, 6, 8, 10 and 24 day p.h. tadpoles (only one 16 and one 18 day p.h. tadpole were exposed to oncomiracidia and both were uninfected at dissection).

If the neotenic form undergoes rapid development there should be a marked difference between these worms and those taking the 'bladder-destined' course. The age of the host at which the neotenic stage is no longer produced would be expected to fall within 10-20 days p.h. (discussed below). The morphological data for the 10 days p.i. worms have been summarised in Table 7.2, with 2 pooled samples (Groups A & B). Those worms recovered from 4-10 day p.h. tadpoles (Group A) were at an equivalent stage of development to the worms from the 24 days p.h. tadpoles (Group B) with comparable standard deviation, although they were recovered from similar burdens and the age of the host spanned the expected transition period.

7.4.3 Neotenic egg production.

As noted above, eggs were first observed in the ootype and recovered from the water at 18 days p.i. However, in the sub-sample screened, commencement of egg production could be delayed until 22 days p.i. The rate of production followed a similar pattern in single and multiple burdens with a gradual increase during the first few days, then reaching a more steady state after 3 to 5 days following first production (Fig.7.8A-C).

Egg production rates were correlated with the final burdens at dissection and worm mortality during the sampling period. Although the worms which became detached were too large to fall through the netting, 16 (14.4%) of the suprapopulation were recovered from the water over a 6 day period and their contribution to previous egg production records noted. However, only 3 of the 20

tadpoles used in the data analysis were known to have lost worms in this manner. There remains the possibility that detached worms may have been eaten by their hosts and hence the record of their contribution to production lost.

Table 7.3 displays the egg production rates of worms in single and multiple burdens for the 3 days prior to dissection. There does not appear to be a density-dependent effect in burdens of 2 worms per host in comparison with single worm burdens but at 3 and above, mean per capita production declines. Morphological analyses of all body features show that they were positively correlated with body length, although there is a degree of variation indicated by Figs.7.3, 7.4 & 7.5. However, body length was chosen to indicate the state of development of each individual worm and this has been correlated with rates of egg production.

The wet body weight of the tadpoles was taken as an indicator of the resource available (all had guts packed with food). Within each tadpole the size of worms showed little variation. Therefore, tadpole body weight (g) divided by the worm burden was used as a measure of the host resource (i.e. blood) available to each worm on that particular tadpole.

Neotenics were found to be smaller at higher burdens and to produce fewer eggs (Figs.7.9 & 7.10). Regression analysis indicates that mean body length is significantly smaller at higher burdens (R^2 = 30.5%, F = 7.89, p = 0.012) and that there was also a significant correlation between body length and egg production (R^2 = 40.9%, F = 12.48 p = 0.002). The mean body length of the

worms was significantly correlated to the amount of host resource available (Fig.7.11; R^2 = 54.8%, F = 21.80, p < 0.001). There was also positive correlation between host resource/worm and their mean egg production/parasite/day. For burdens of 3-9 parasites/host (Fig.7.12) the relationship was clear (R^2 = 47.7%, F =11.87 ,p = 0.004). However, if single and double burdens are included (Fig.7.13) though still significant (R^2 = 26.6%, F = 6.52, p = 0.02) there does appear to be a threshold level (approximately 0.2 g/parasite) above which host resources are no longer a limiting factor. There was no significant correlation between the age of the neotenics, 22-28 days at dissection and their mean body length (Fig.7.14; R^2 = 0.6%, F = 0.11, NS) or mean egg production.

7.5 Discussion.

The pattern of establishment and egg production of neotenic *P.nearcticum* closely resembles that of *P.umthakathi* and *P.australis* (see Kok, 1990; Kok & DuPreez, 1989). Kok (1990) achieved an overall invasion success of 76.3% with a mean intensity of 2.1, although infection doses and conditions were at variance with this study. Previous studies have recorded that 84% of *P.integerrimum*, 97.4% of *P.australis* and over 90% of *P.umthakathi* were recovered from the closed side of the branchial chamber (Gallien, 1932a; Kok & DuPreez, 1989; Kok, 1990), however, 94.9% of *P.nearcticum* established and remained on the open side of the chamber. The hatching of *P.nearcticum* eggs 9 to 11 days after release at constant temperature (20.6 +_0.8^{0}C) is of the same order as the 11 to 13 day period before hatching 'at room temperature' recorded by Paul (1938).

The detachment and loss of neotenic worms from the branchial chambers of host tadpoles has been reported by a number of authors (Kok, 1990; Murith, 1981b; Savage, 1950). This loss of parasites could be due either to the accidental detachment of worms, which are then washed out by the respiratory current via the spiracle or to host immune responses. Kok & DuPreez (1989) stated that mature neotenics of *P.australis* 'invariably' attach themselves to the front limb buds which they suggested provided a more rigid site; however, very few *P.nearcticum* made this transition. The possibility of detachment is increased as *P.nearcticum* do not migrate to the closed side of the branchial chamber. Williams (1960) stated that 'bladder-destined' *P.integerrimum* may possess posterior, or posterior and middle suckers in comparison to the neotenic which has all three sucker pairs. If this is mirrored by *P.nearcticum*, as the haptor becomes proportionally smaller in comparison to overall body length (Fig.7.3), then the haptor is larger (in proportion to body length) for the 'bladder-destined' form than the neotenics. Thus on a simple mechanistic level the haptor of 'bladder-destined' forms will facilitate a proportionally larger area of attachment to body size. In addition, neotenics must also support the extra weight of a fully functioning reproductive system and the host blood in their guts. Therefore, if it is overall body weight that, under the influence of the respiratory current, pulls the worms off the gill filaments neotenics will be at a relative disadvantage.

Alternatively parasites may be attacked and killed by host-mediated responses. This may include acquired immunity due to repeated exposure to infective stages as well as localised inflammation of tissue. Williams (1960) stated that 'bladder-

destined' *P.integerrimum* were blood feeders and so vulnerable to attack by the host's immune system. Murith (1981b) proposed that 'bladder-destined' forms were antigenically different from neotenics, which may explain the low mortality of 'bladder-destined' forms of *P.integerrimum* from *Rana temporaria* reported by Combes & Bremond (1988). The crowded distribution of *P.nearcticum* on the gill filaments and their proximity to the spiracle is the most probable cause of the loss of neotenics from the branchial chamber. The detachment of worms is an intriguing aspect of neotenic dynamics and will have a major influence on their contribution to transmission.

The growth of *P.australis* documented by Kok & DuPreez (1989) and *P.marmorati* recorded by Van Niekerk (1992) is similar to that recorded for *P.nearcticum*. Although sample size is small prior to 22 days p.i., it appears that the rate of growth of total body length increases between 12 and 20 days p.i. for *P.nearcticum*, slowing at neotenic maturity. The increase in pharynx dimensions are again comparable between *P.nearcticum* and *P.marmorati* both showing low coefficients of variation. The drop at 16 days p.i. in Figs 7.3 & 7.4 is most probably due to the small sample of 3 worms which all contracted during fixation. Fig.7.2D illustrates one of these worms, where there is no clear margin between the body and haptor and the ovary is bent. The apparent decrease in body, haptor and to some extent, pharynx length at 26 days p.i. can be related to host size and worm burden. The hosts of specimens collected at 24 days p.i. weighed on average 12% more than those at 26 days p.i. The tadpoles harbouring worms at 28 and 30 days p.i. were also lighter than those at 24 days p.i. In addition, worm

burdens were also, on average, higher at 26 to 30 days p.i.

The differential development of sucker pairs has also been recorded by Kok & DuPreez (1989) and Van Niekerk (1992) for *P.australis* and *P.marmorati* respectively. Subtle differences in development can be noted, *P.australis* suckers are close to parity at 20 days p.i. whereas both *P.marmorati* and *P.nearcticum* still show inequality even at 30 days p.i. This provides a useful means of age determination in the early stages of development. In addition, the suckers of *P.nearcticum* are marginally smaller than those of both African species.

The alimentary tract of *P.australis* possesses no anastomoses, in *P.marmorati* their existence was unclear, which contrasts with the highly diverticulated and anastomosed gut of *P.nearcticum* at 14 days p.i. The development of the reproductive system begins with the emergence of the deeply staining ovary which is comparable for the above 3 species at 6-8 days; however, the whole system becomes distinct at different rates, *P.australis* at 10 days, *P.marmorati* at 12 days and *P.nearcticum* at 14 days p.i. Genital spines are 12μm in *P.australis* at 10 days, 23μm in *P.marmorati* at neotenic maturity and 16μm in *P.nearcticum*.

The descriptions of neotenic *Polystoma* (Kok & DuPreez, 1989; Maeder, 1973; Murith, 1981a & b; Van Niekerk; 1992) show some variation with only the marginal hooklets providing species specific features. The documentation of neotenic development and morphology may provide further information as to the systematics and diversity within the genus.

Gallien (1934) proposed that it was the age of the tadpole in conjunction with the quality and quantity of the blood ingested by *P.integerrimum* which determined the life-cycle path taken. As noted in the Introduction, Murith (1981b) found that the age of the host at which the change from neotenic to 'bladder-destined' form was imprecise. Further investigation by Murith (1982) showed that if tadpoles were prevented from metamorphosing by chemical treatment then *P.integerrimum* considered those tadpoles as identical hosts to young tadpoles, indicating the reproductive strategy depends upon biochemical aspects of the host.

The data gathered for *P.nearcticum* as regards the timing of the changeover from neotenic to 'bladder-destined' courses are incomplete but it is interesting to note that the worms recovered from the 24 days p.h. tadpoles were of comparable state of development to those from 4-10 days p.h. If the worms take 18 days to reach maturity, as has been recorded at 20°C, then neotenics infecting tadpoles 24 days p.h. would not be able to contribute to infection prior to host metamorphosis, if this occurs as early as 35 days after oviposition (recorded for *H.chrysoscelis* in Tennessee by Ritke *et al.*, 1990). If metamorphosis is delayed or recently hatched tadpoles are infected, the role of the neotenic in transmission is further reduced as there is a lag of 2-3 days before eggs are produced at the peak rate. However, sample sizes are small and further investigation is required before firm conclusions can be made.

Kok & Seaman (1987) and Kok & DuPreez (1989) stated that the fecundity of *P.australis* neotenics was of great importance as they were responsible for the

majority of reproductive output which successfully infected the host population. Up to 90% of tadpoles harboured neotenic *P.australis* which have a possible reproductive life-span of 40-50 days producing eggs at a rate of 10-20 eggs/parasite/day. The neotenic form therefore becomes important in transmission as the adult hosts make infrequent visits to waterbodies, reducing the contribution of the adult worms.

It appears, therefore, that the role of neotenics between species is variable, Tinsley (1983) indicated that although the fecundity of *P.integerrimum* could be augmented by neotenics, Combes (1968) had reported that this form was rare in certain areas. Combes (1968) related this to the difference in hatching times between the parasite and host. In colder regions the difference in hatching is increased from 5 days at 25^{0}C to over 50 days at 5^{0}C. Therefore, at lower temperatures the neotenic stage would have a reduced role in transmission.

Neotenics may produce a continuous 'trickle' infection but their role in transmission may be elevated if there have been no host breeding nights close to the metamorphosis of a cohort of tadpoles. Without the input of adult-derived eggs 9 to 11 days earlier, the chance to infect those tadpoles already present in the pond with 'bladder-destined' forms could be missed. Savage (1950) proposed that the major role of the neotenic cycle was to elevate infection levels just prior to host metamorphosis. Although Paul (1938) found over 30 eggs in all stages of development, including fully formed larvae in the uterus of a single, adult *P.nearcticum*, ovoviviparity has not been demonstrated for this monogenean.

Therefore, once a tadpole has metamorphosed the chance to infect that individual has been lost.

It was hoped to sample tadpoles from natural surroundings in order to document the expected waves of infection each age cohort would experience from the punctuated host breeding nights. This would have not only documented the developmental rate in nature, but establishment and life-cycle course chosen by oncomiracidia invading the gills of tadpoles which are already infected. This was not possible as all the host breeding pairs were removed from the main study site prior to oviposition by co-workers.

Kennedy (1975) in his review of competition noted 'as the parasite population increases, so does the pressure on the limited resources, leading to intra-specific competition. Competition is accentuated by the restriction of the parasites to a preferred site'. An important finding in this study is that of density-dependent egg production similar to that found by Kok (1990) for *P.umthakathi*. However, in the present study, the ratio chosen to indicate host resource (at levels of less than 0.2g wet weight/parasite) has been shown to have a significant relationship to the production of eggs by neotenic *P.nearcticum*. It was not possible to base the analysis solely on burdens of 1-2 worms/host due to insufficient sample size within the 35 replicates set-up (which would have avoided competitive effects between parasites). The remaining unexplained variation in the regression analysis is most probably due to those competitive interactions in burdens of 3-9 worms/host. Above 0.2g wet weight/parasite, other factors such as individual heterogeneity

may also influence production. Jackson & Tinsley (1988a) documented variable egg production of *Protopolystoma xenopodis* in single burdens, which was maintained after the transfer to another host, indicating a genetically predetermined reproductive ability.

The daily egg output documented in Figs.7.8 A-C show the degree of variation for neotenic *P.nearcticum*. This can be related to host factors, for example, the lowest rate of production from burdens of 5 worms (Fig.7.8B) was from worms infecting the smallest and lightest tadpole (number ii). The separation of resource and innate reproductive ability is not possible without further studies utilising eggs derived from the same adult worm, which has not been exposed to the possibility of cross-fertilisation.

Kok & DuPreez (1989) found 'no apparent correlation between the age and size of mature neotenics' at 29 to 66 days p.i. A similar trend was found for *P.nearcticum*, although the range of ages was smaller. The age of mature neotenics did not influence their rate of egg production, however, a significant correlation was found for egg production, based on their body length (Fig.7.10; $F = 12.48$,$p = 0.002$). This finding, combined with the data presented in Figs.7.3 & 7.8, suggests that neotenics attain a particular size at maturity (influenced in part by host size and infrapopulation density) which is a determinant of their maximum egg production rate.

The drain upon host resources has implications for worms establishing on young tadpoles, as well as those host species which have small larval stages. Kok & Seaman (1988) detailed the larval development of *Natalobatrachus bonebergi* (the host of *P.umthakathi*), and the snout to tail-tip length is of the same order as *H.versicolor*. Thus, both cases of density-dependent egg production by neotenic *P.nearcticum* and *P.umthakathi* occur in tadpoles of a comparable size. Kok & DuPreez (1989) found no crowding effect with *P.australis* which infects a comparatively large host, indicating that host resource is a factor in neotenic *P.nearcticum* transmission dynamics. However, Savage (1950) indicated that *P.integerrimum* did not adversely affect each other in multiple burdens nor the host tadpoles. In addition, an inverse relationship between worm burden and egg production has been documented for adult *P.integerrimum* (see Combes, 1972) and *Protopolystoma xenopodis* (see Jackson & Tinsley, 1988a) which both inhabit comparatively massive hosts. The survivorship of the host becomes of concern if 'bladder-destined' forms inhabit the same cohort of tadpoles.

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	Mean	S.D.	Paul (1938) Mean
Body length	2.81	0.58	2.04
Body width	0.79	0.16	0.45
Haptor length	0.58	0.09	-
Haptor width	1.05	0.19	-
Haptor length/Body length	0.207	-	-
Pharynx length	0.12	0.01	-
Pharynx width	0.11	0.01	0.10
Sucker pair 1	0.16	0.01	-
Sucker pair 2	0.15	0.02	-
Sucker pair 3	0.14	0.02	0.12
Ovary length	0.61	0.09	0.41
Ovary width	0.15	0.03	-
Egg length	0.300	0.020	0.30
Egg width	0.150	0.010	0.15
Genital spine length	16μm	0.5μm	-

Table 7.1 Body dimensions of 20 mature neotenic *Polystoma nearcticum* (30 days p.i.) recovered from experimentally infected *Hyla versicolor* tadpoles. Specimens recovered from burdens of 4 parasites/host (n = 14) and 6 parasites/host (n = 6). All measurements in mm, except where indicated. Data from Paul (1938) based on 10 mature specimens.

	A.			B.		
	Mean	S.D.	n	Mean	S.D.	n
Body length	0.53	0.10	13	0.51	0.09	10
Body width	0.19	0.03	12	0.20	0.02	11
Haptor length	0.15	0.04	13	0.15	0.04	7
Haptor width	0.23	0.04	14	0.24	0.05	7
Haptor lth/Body lth	0.28	-	-	0.28	-	-
Pharynx length	0.05	0.01	20	0.05	0.01	12
Pharynx width	0.05	0.01	20	0.06	0.01	12
Sucker pair 1	0.07	0.01	14	0.07	0.01	8
Sucker pair 2	0.04	0.01	11	0.05	0.01	8
Ovary length	0.15	0.02	15	0.14	0.02	9
Ovary width	0.08	0.01	15	0.08	0.01	9

Table 7.2 Body dimensions of *P.nearcticum* (10 days p.i.) recovered from:

Group **A.**) 4 to 10 days p.h. *Hyla versicolor* tadpoles (pooled sample of parasites, n = 22 from burdens of 1-4 parasites/host).

Group **B.**) 24 days p.h. *H.versicolor* tadpoles (n = 12 from burdens of 1-6 parasites/host).

All measurements in mm.

Worm Burden	Total eggs/host/day 1	2	3	Mean (eggs/parasite/day)	S.D.
1	10	10	9		
1	9	10	8	9.33	0.62
2	24	30	33		
2	25	22	30		
2	25	15	23	12.57	1.29
3	34	39	40		
3	33	33	23		
3	23	30	42	11.00	0.73
4	37	23	31	7.63	1.43
5	15	15	9		
5	40	42	51		
5	20	18	16		
5	23	25	29		
5	47	50	47	5.96	0.12
6	16	13	14		
6	41	62	56		
6	48	49	46		
6	78	74	70	7.90	0.29
7	43	64	37	6.83	1.64
9	44	39	-	4.60	0.30

Table 7.3 Egg production of neotenic *Polystoma nearcticum* in burdens of 1-9 worms per *Hyla versicolor* tadpole. Counts taken on 3 consecutive days during peak production (18-28 days p.i.).

7.7 Legends.

Fig.7.1 Distribution of *Polystoma nearcticum* on the internal gills of *Hyla versicolor* tadpoles 2-30 days p.i.

Fig.7.2 Size and morphological changes during the development of *Polystoma nearcticum* towards neotenic maturity at 20.6°C (+_0.8°C). **Scale bar:** 0.8 mm. **A**: 8 days; **B**: 10 days; **C**: 14 days; **D**: 16 days; **E**: 18 days p.i.

Fig.7.3 Relationship between total body length and haptor length during the development of neotenic *Polystoma nearcticum* 6-30 days p.i. (n = sample size).

Fig.7.4 Pharynx length of neotenic *Polystoma nearcticum* 6-30 days p.i. (n = sample size).

Fig.7.5 Development of the haptoral sucker pairs of neotenic *Polystoma nearcticum* 2-30 days p.i. (S1 = posterior pr., S2 = middle pr. and S3 = anterior pr., n = no. of worms analysed).

Fig.7.6 *Polystoma nearcticum*, sexually mature neotenic form 30 days p.i. The positions of selected organ systems have been noted. Extent of the vitelline system indicated by stippled regions [illegible] and gut [illegible]. **Abbreviations: an**, anastomose; **eg**, egg; **gc**, gut caecum; **hp**, haptor; **os**, oral sucker; **ov**, ovary; **ph**, pharynx, **suc**, haptoral sucker. **Scale bar:** 0.5 mm.

Fig.7.7 *Polystoma nearcticum*, detail of the reproductive system of sexually mature neotenic form 30 days p.i. **Abbreviations: eg**, egg; **gs**, genital spines; **od**, oviduct; **ol**, ovovitelline canal; **ov**, ovary; **ts** & [illegible], testis; **vd**, vas deferens; **vc**, vitelline canal. **Scale bar:** 0.3 mm.

Fig.7.8A *Polystoma nearcticum* neotenics: mean per capita egg production in single worm burdens, i) & ii) and 3 (iii) and 4 (iv) worms/host respectively.

Fig.7.8B *Polystoma nearcticum* neotenics: mean per capita egg production in burdens of 5 parasites/host from four individual tadpoles (i to iv).

Fig.7.8C *Polystoma nearcticum* neotenics: mean per capita egg production in burdens of 6 (i to iii) and 7 (iv) parasites/host from four individual tadpoles.

Fig.7.9 Relationship between mean body length of mature, egg producing neotenic *P.nearcticum* and worm burden host (R^2 = 30.5%, F = 7.89, p = 0.012).

Fig.7.10 Relationship between mean number of eggs per parasite produced per 24 h for neotenic *P.nearcticum* and mean body length (R^2 = 40.9%, F = 12.48, p = 0.002).

Fig.7.11 Relationship between mean body length of mature, egg producing neotenic *P.nearcticum* and host resource (host wet body weight / worm burden) (R^2 = 54.8%, F = 21.80, $p < 0.001$).

Fig.7.12 Relationship between mean number of eggs/parasite/24 hours for mature neotenic *P.nearcticum* and host resource in burdens of 3 to 9 worms per host (R^2 = 47.7%, F = 11.87, p = 0.004).

Fig.7.13 Relationship between mean number of eggs/parasite/24 hours for mature neotenic *P.nearcticum* and host resource in burdens of 1 to 9 worms per host (R^2 = 26.6%, F = 6.52, p = 0.02).

Fig.7.14 Relationship between mean body length of mature, egg producing neotenic *Polystoma nearcticum* and age post infection (R^2 = 0.6%, F = 0.11, NS).

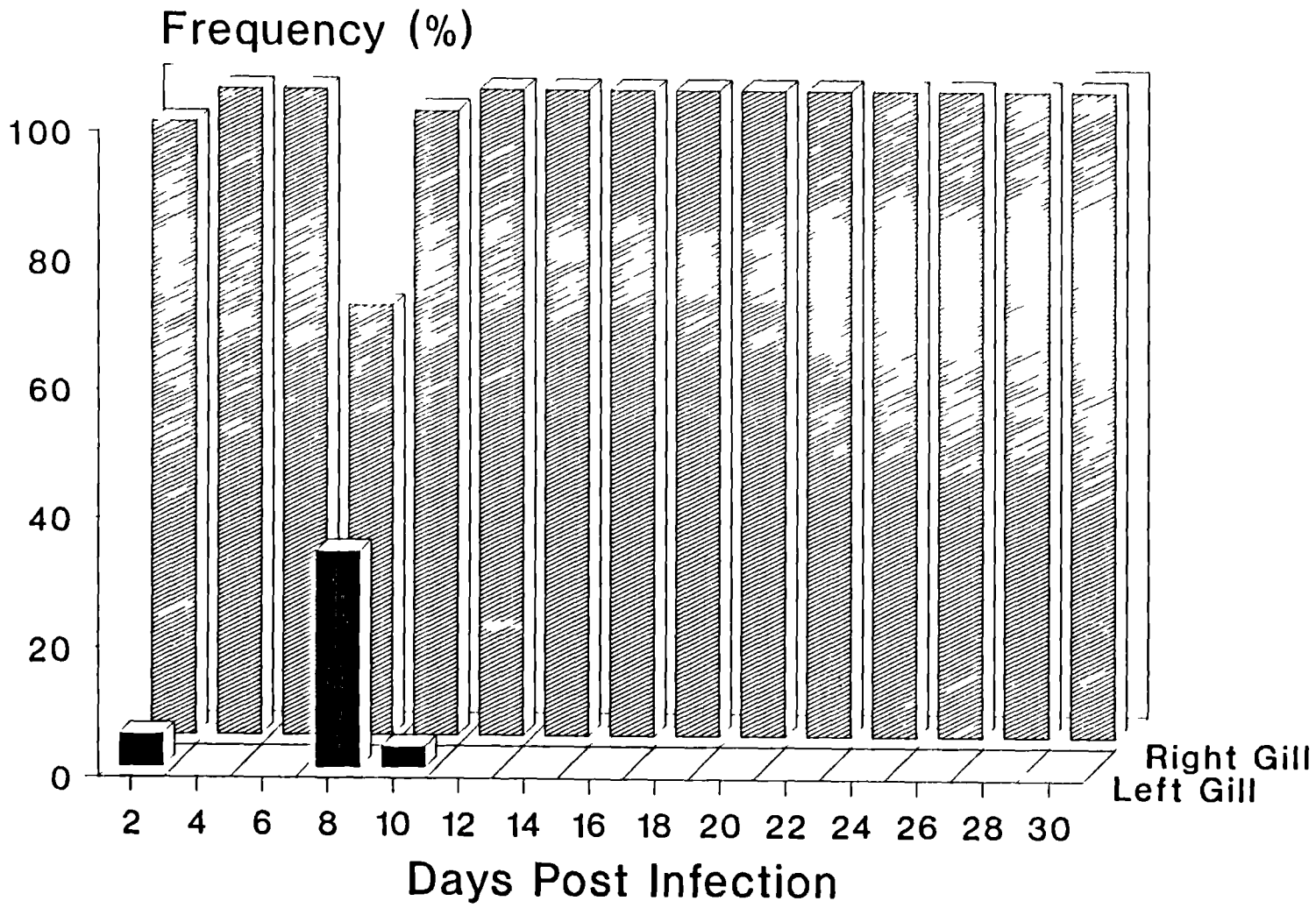
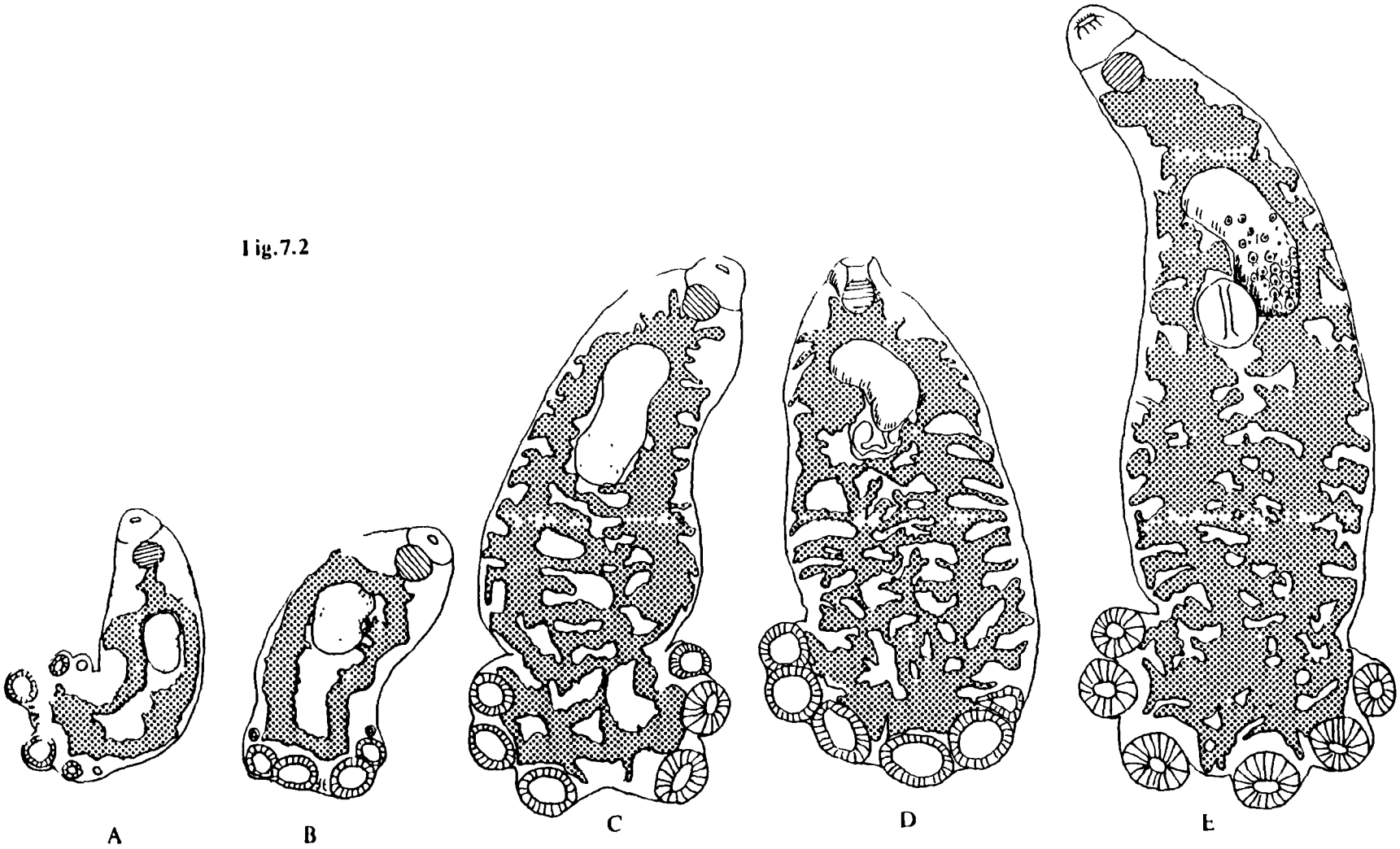

Frequency (%)
100
80
60
40
20
0
2
4
6
8
10
12
14
16
18
20
22
24
26
28
30
Days Post Infection
Right Gill
Left Gill

Fig.7.1

Fig.7.2



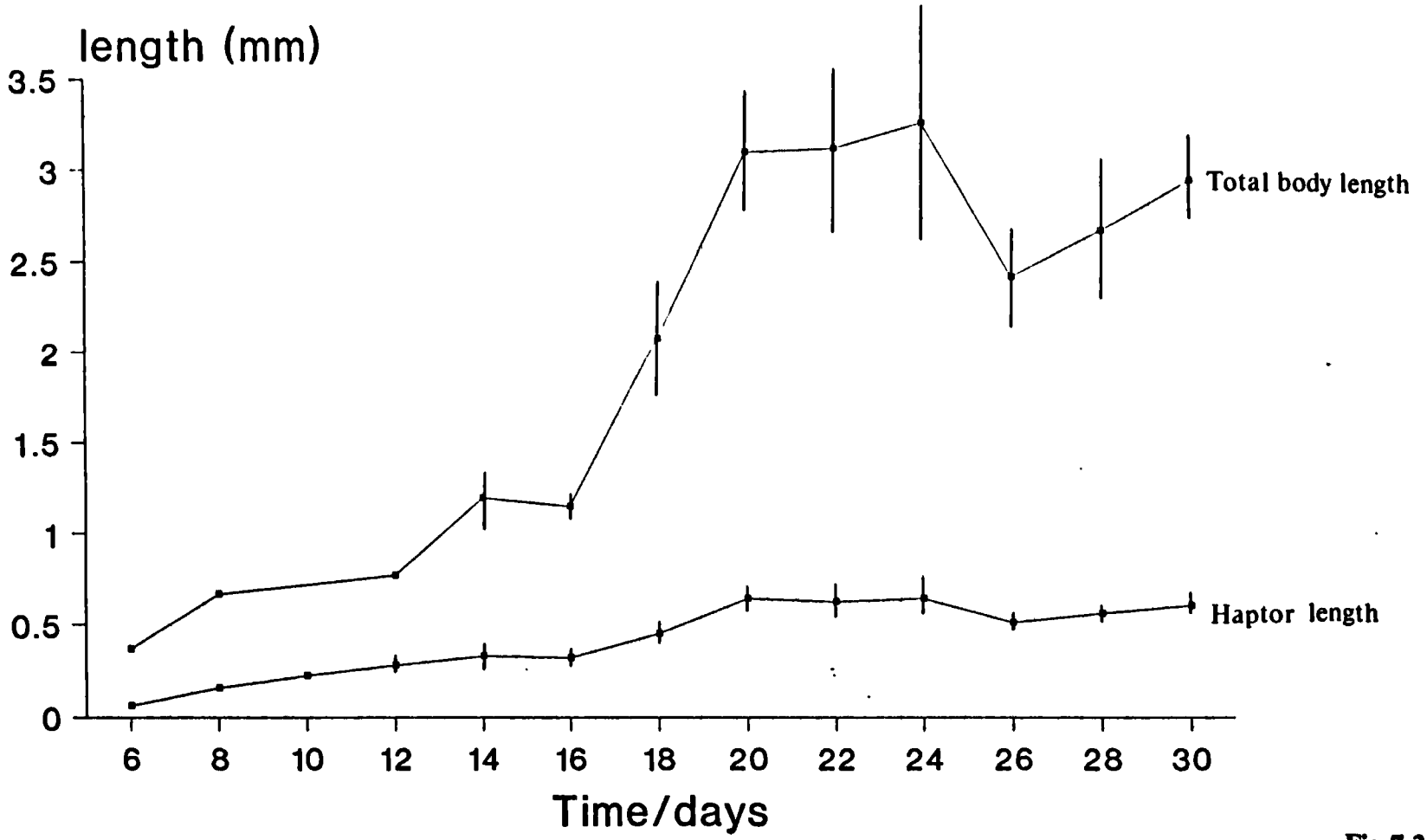

length (mm)
3.5
3
2.5
2
1.5
1
0.5
0
Total body length
Haptor length
6
8
10
12
14
16
18
20
22
24
26
28
30
Time/days
n
1
1
1
2
8
3
7
8
15
17
13
17
11

Fig.7.3

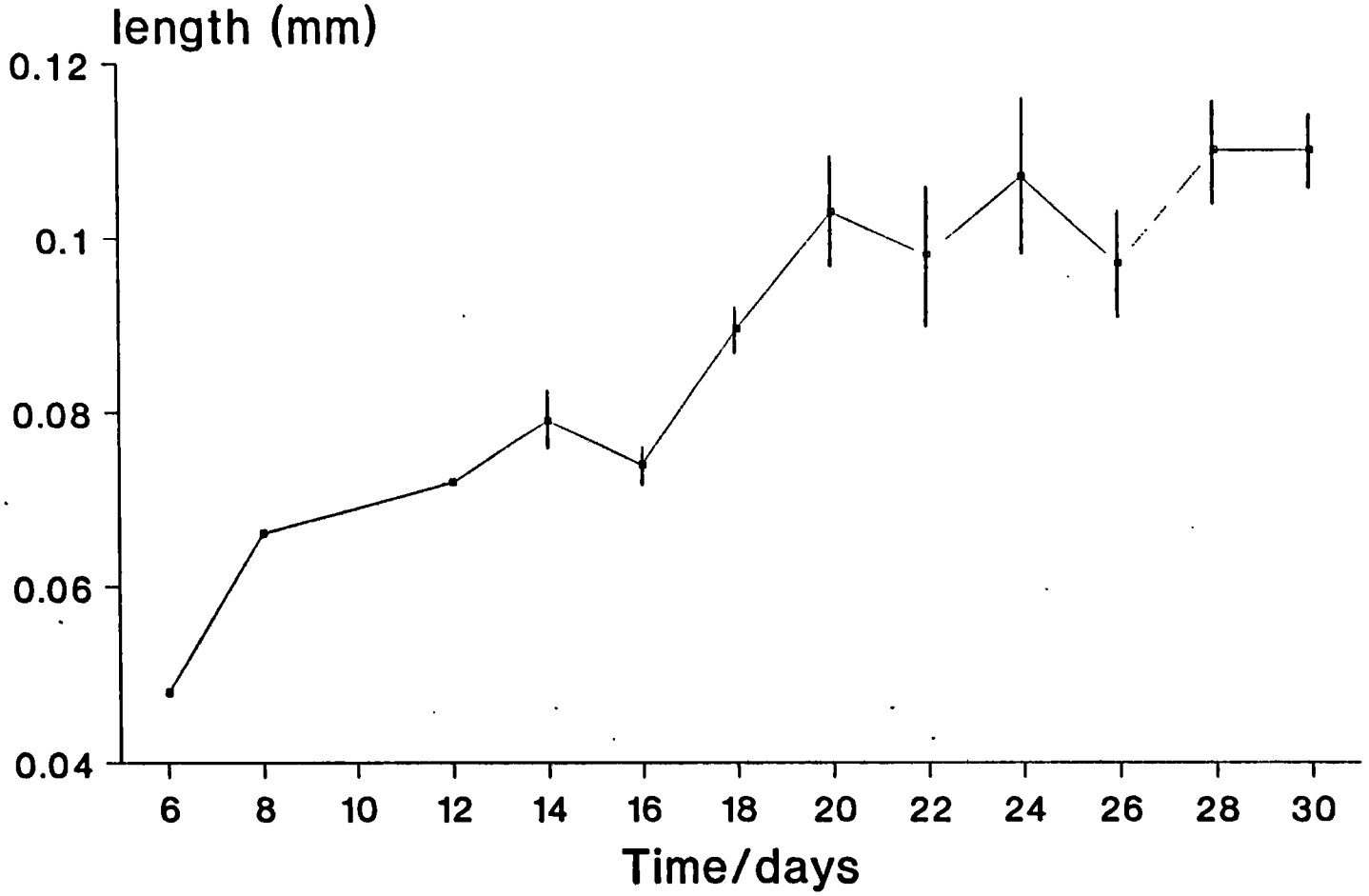


Fig.7.4

n	3	1	0	1	7	2	4	6	16	14	13	17	12

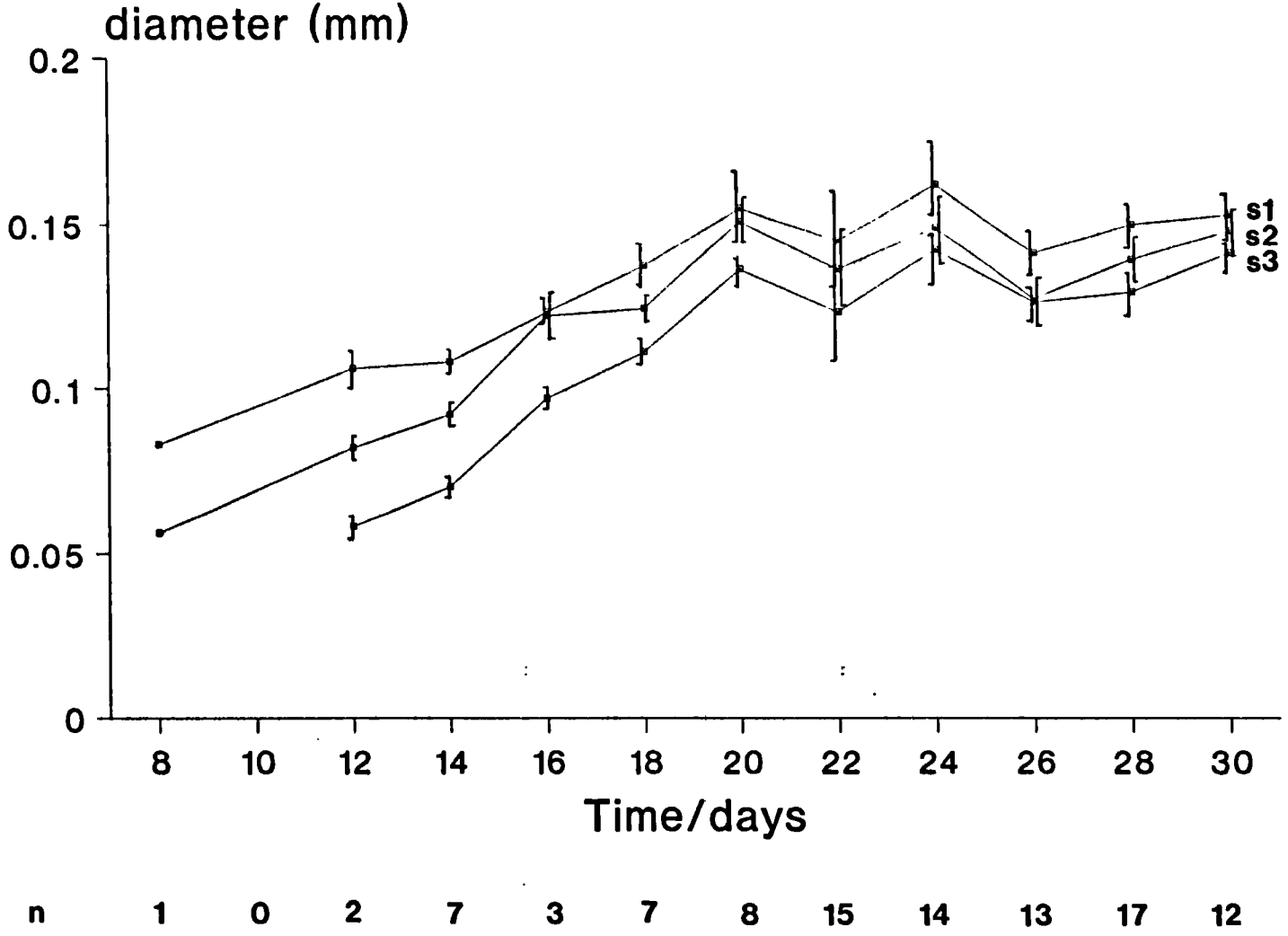

diameter (mm)
0.2
0.15
0.1
0.05
0
s1
s2
s3
8
10
12
14
16
18
20
22
24
26
28
30
Time/days
n
1
0
2
7
3
7
8
15
14
13
17
12

Fig.7.5

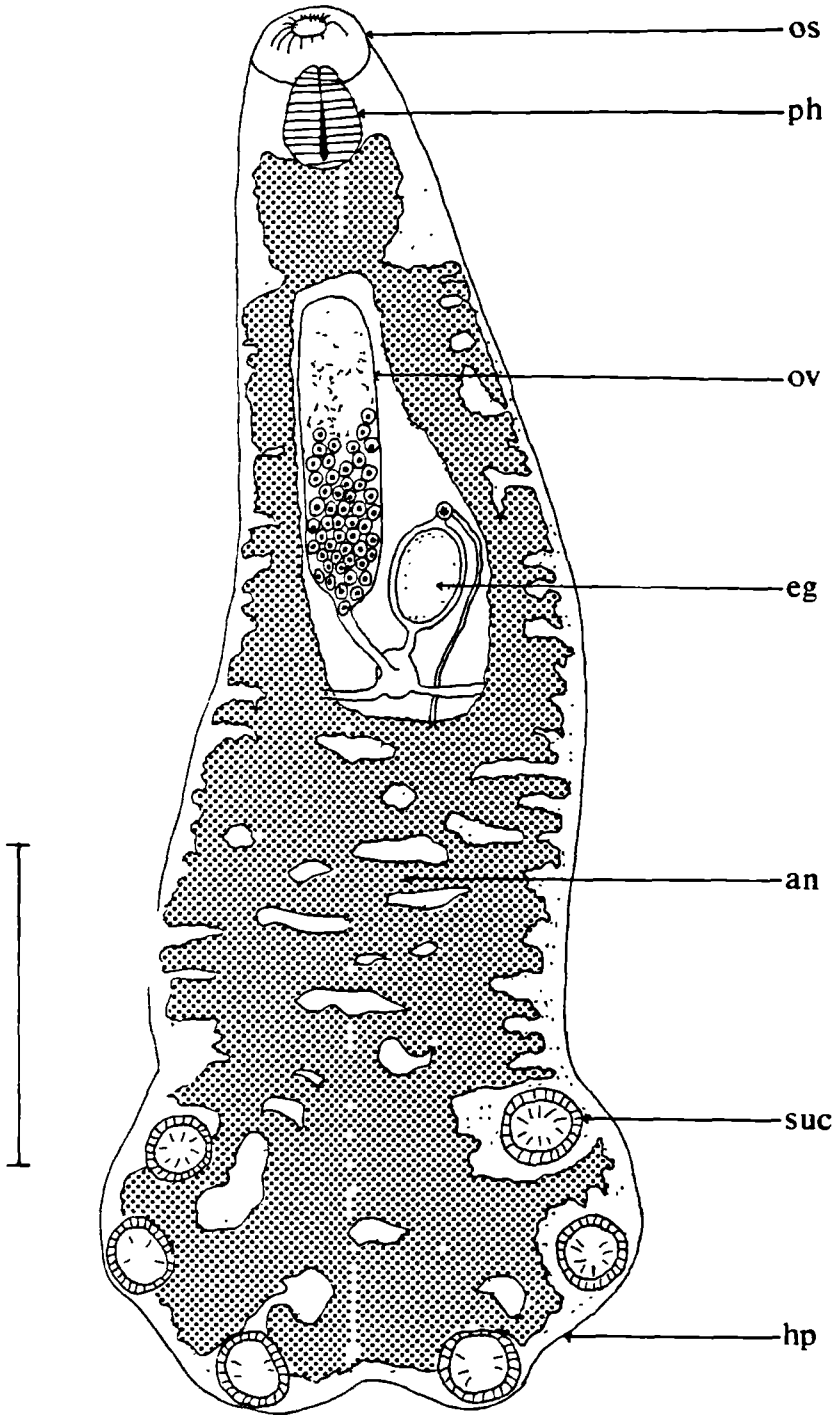


Fig.7.6

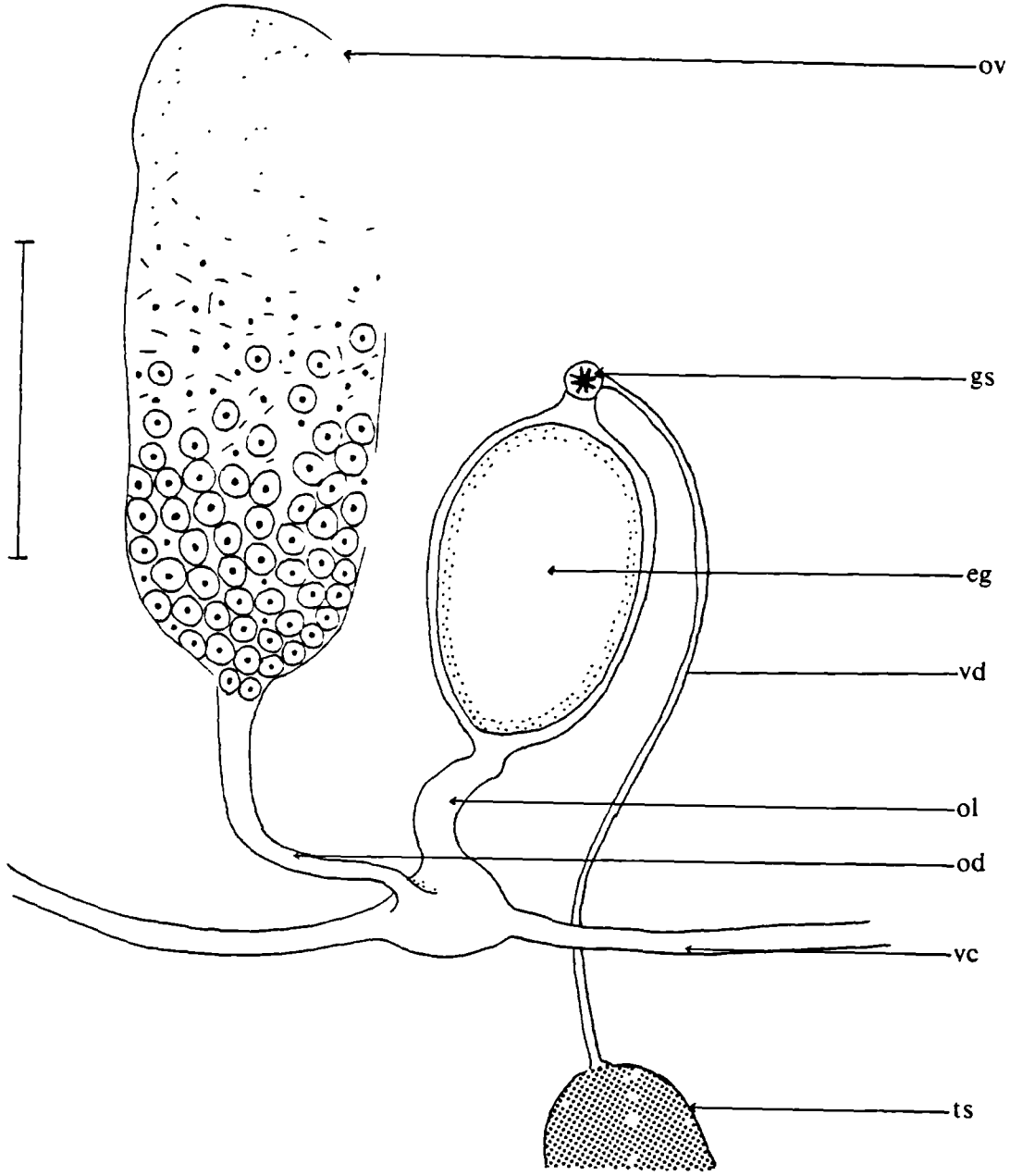


Fig.7.7

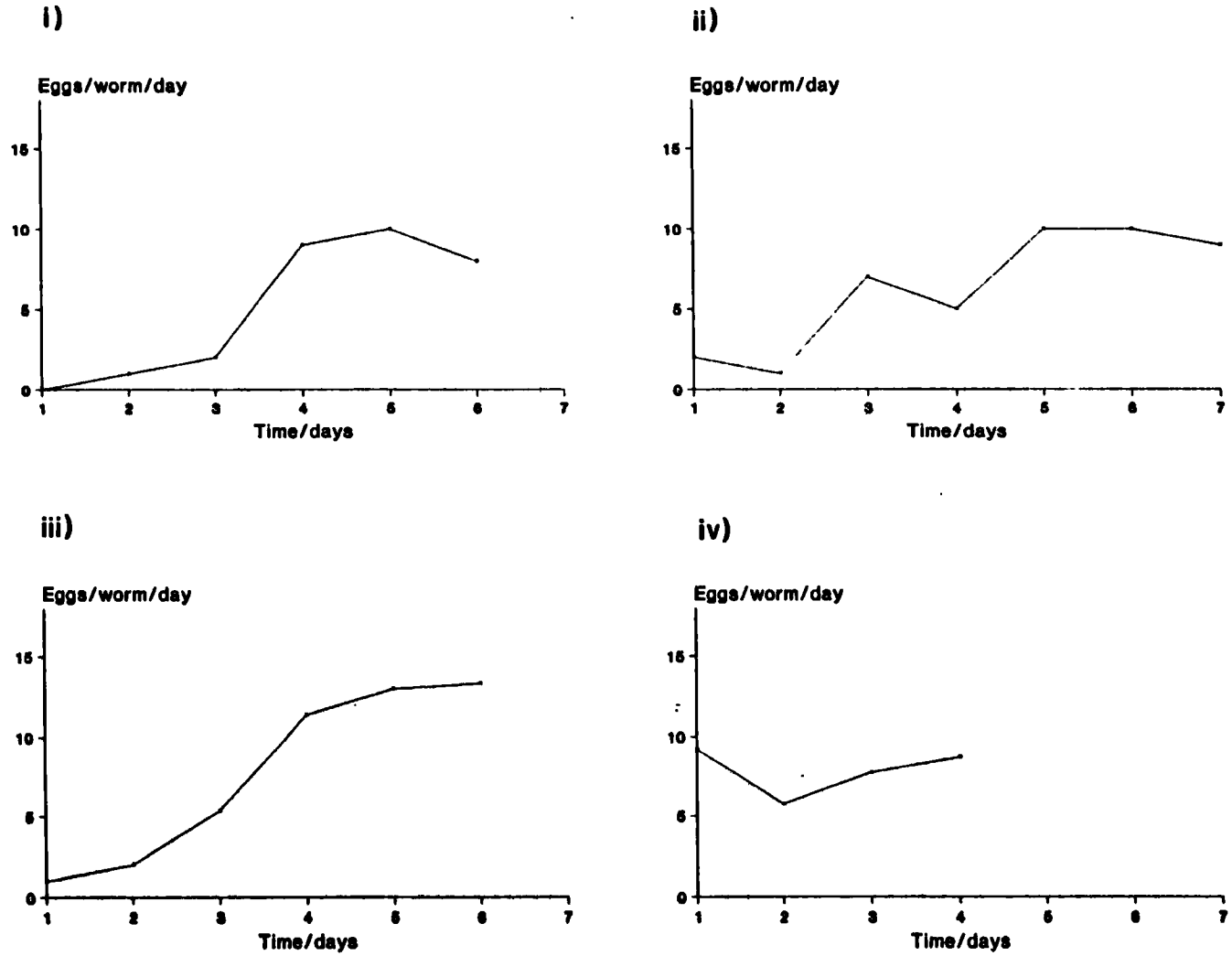

i)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
ii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iv)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days

Fig.7.8A

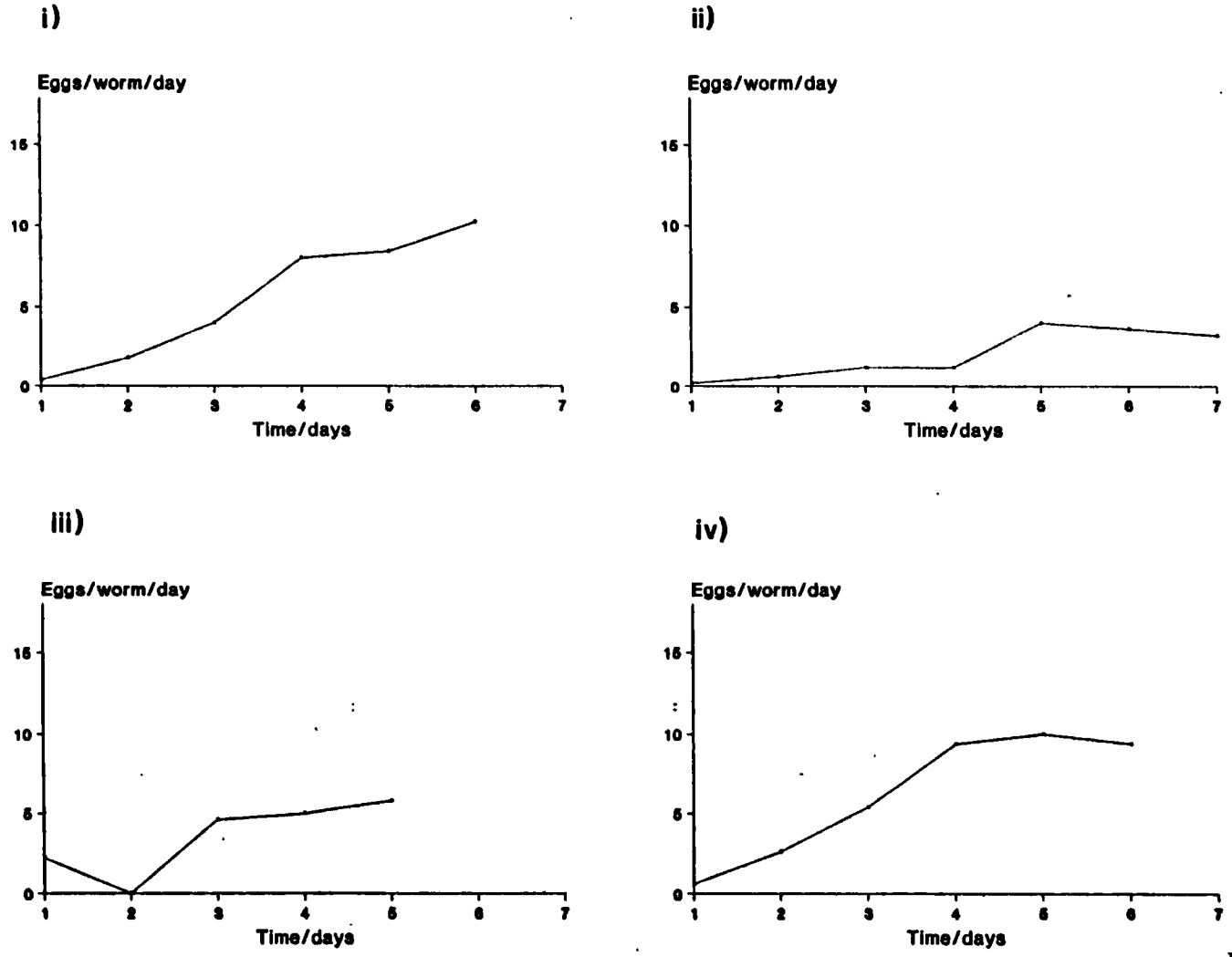

i)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
ii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iv)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days

Fig.7.8B

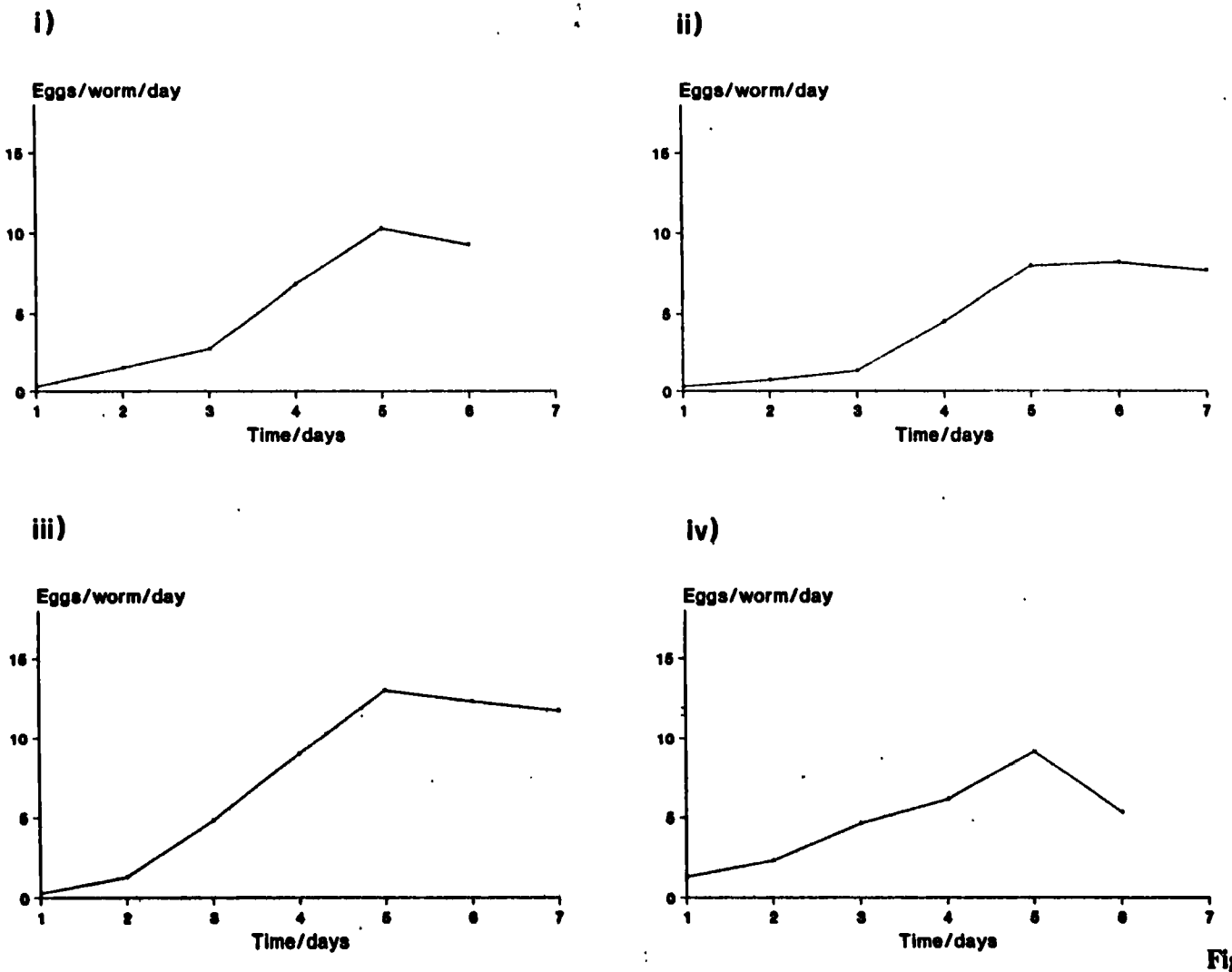

i)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
ii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iii)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days
iv)
Eggs/worm/day
15
10
5
0
1
2
3
4
5
6
7
Time/days

Fig.7.8C

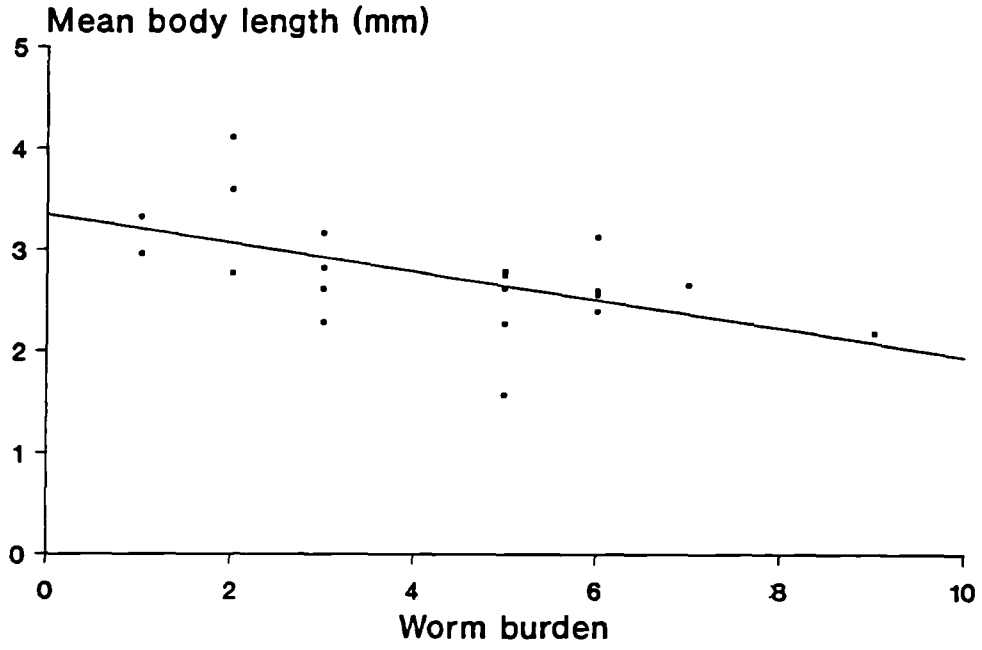


Fig.7.9

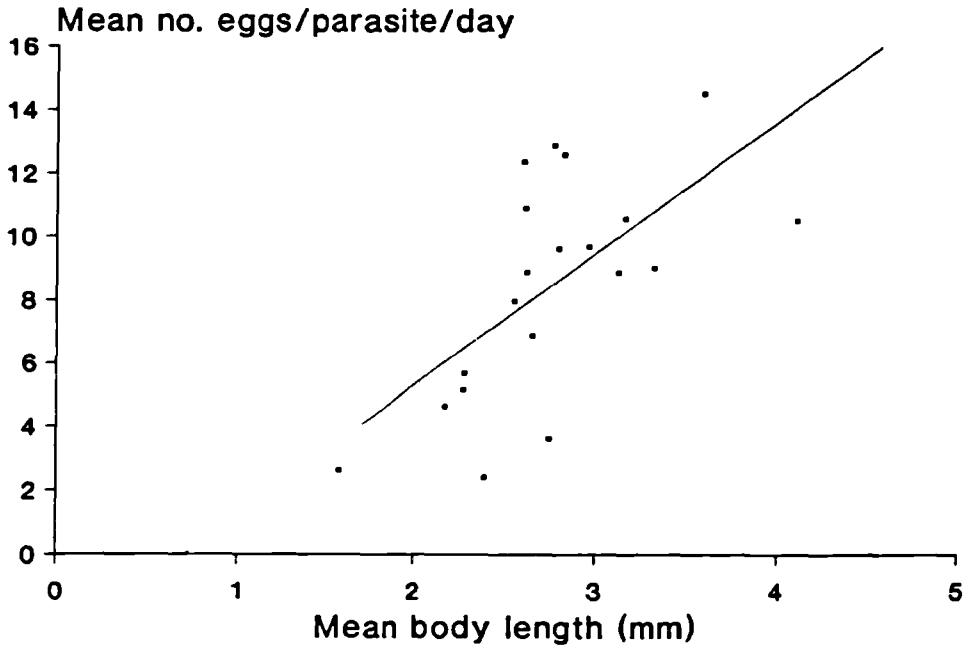


Fig.7.10

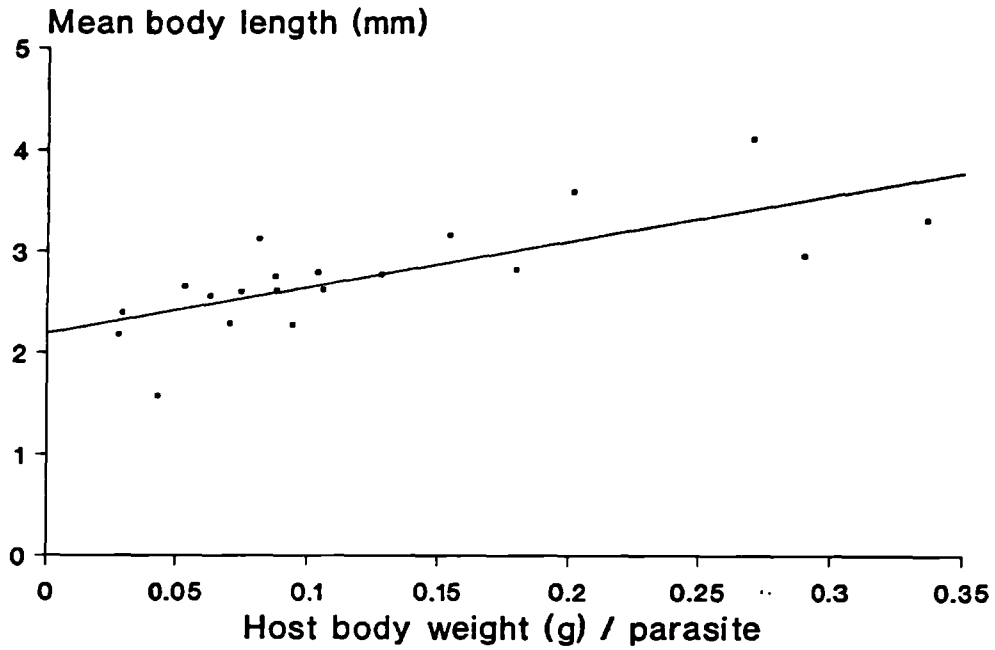


Fig.7.11

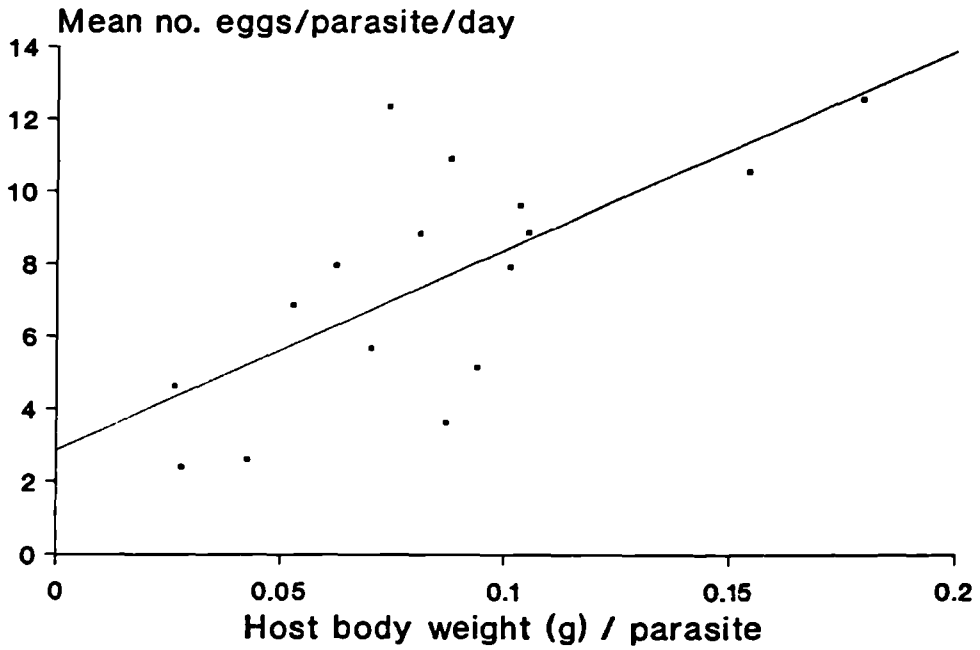


Fig.7.12

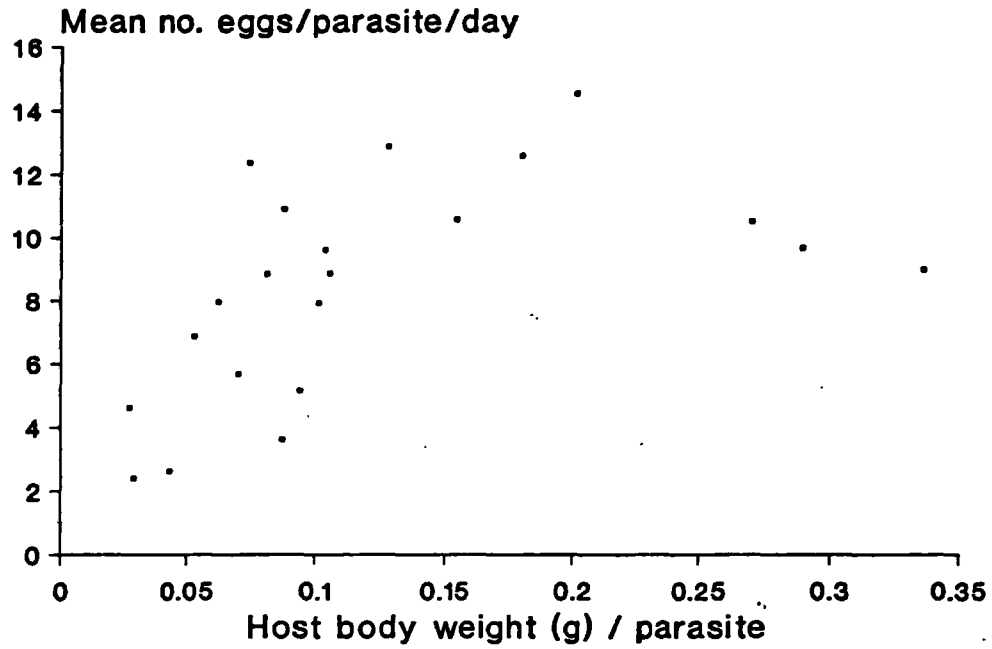


Fig.7.13

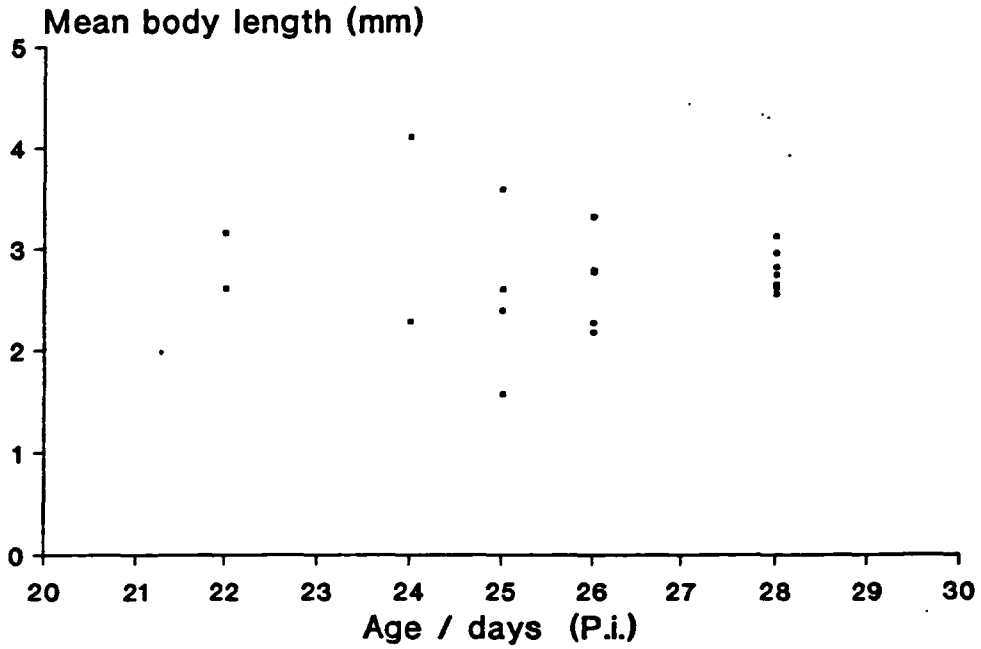


Fig.7.14

Chapter 8.

A Parasitological Survey of the Gray Treefrogs *Hyla chrysoscelis* and *H.versicolor* (Anura:Hylidae) from the United States.

8.1 Abstract.

The Gray treefrogs, *Hyla chrysoscelis* (2n=24) and *H.versicolor* (2n=48), are common to the woodlands of the eastern and mid-western United States. A total of 92 *H.chrysoscelis* and 90 *H.versicolor* were collected from areas of sympatry and allopatry at 12 locations in 9 States. These phenotypically identical species were distinguished by flow cytometry and mating call. A full parasitological survey revealed that both hosts shared a virtually identical fauna. Representatives of 5 parasitic groups were found: *Polystoma nearcticum* (Monogenea); *Megalodiscus temperatus* and *Clinostomum complanatum* (Digenea); *Cylindrotaenia americana* and protocephalid larvae (Cestoda); *Cosmocercella haberi*, *Dorylaimus sp.*, *Physaloptera sp.*, *Rhabdias ranae* and unidentified larval cysts (Nematoda); *Nyctotherus sp./Balantidium sp.* and *Opalina sp.* (Protozoa). A number of new host and/or locality records are described.

The four most common parasites, *C.haberi*, *Dorylaimus sp.*, *Physaloptera sp.* and *P.nearcticum* recovered from the three major sampling sites, formed the basis of statistical analyses. No significant relationship between parasitic infection and a suite of host factors was found for either host. Both *C.haberi* and *Dorylaimus sp.* showed significant differences ($p < 0.05$) in their distribution between host species at different localities but only *Dorylaimus sp.* had a significantly different ($p < 0.05$) distribution between host species in sympatry and allopatry (individual Mann-Whitney tests). These variations are discussed in relation to host ploidy, ecology and geographical range, as well as the transmission processes employed by the different parasites.

8.2 Introduction.

Only certain aspects of the biology of *Hyla chrysoscelis* and *H.versicolor* have been documented in the literature; as noted in Chapter 6, these anurans have been the subject of detailed research concerning the origins of their polyploid complex. In addition, they have been widely used as models for establishing the criteria for female sexual selection (Gerhardt & Doherty, 1988; Klump & Gerhardt, 1987; Morris, 1989; Morris & Yoon, 1989; Sullivan & Hinshaw, 1992). However, surprisingly little is known about their life history traits, both during and after the breeding season, behaviours which might influence their exposure to parasite infective stages.

It is known that *H.chrysoscelis* and *H.versicolor* are typically found in small wooded plots near water courses. They are nocturnal, feeding on invertebrate prey either on the ground or in the arboreal vegetation. During daylight hours they may be found under leaves, bark or in hollows. Ritke & Babb (1991) observed *H.chrysoscelis* from early autumn to early winter in Tennessee. Characteristic behaviour centred on resting sites in tree knotholes, which were sometimes filled with water or debris. The frogs rarely fed in the daytime but emerged at night, waiting for invertebrate prey to approach before striking. Site fidelity was strong with individuals generally returning to the same hole each night.

During periods of cold weather, *H.versicolor* survives freezing temperatures by utilising stored glycerol as a cryoprotectant (Storey & Storey, 1985). The sites where the frogs hibernate through the winter months is obscure (Johnson, 1987)

but it has been postulated that they hibernate under the soil surface, under leaves, tree roots or rocks (Froom, 1982 cited by Storey & Storey, 1987). Ritke & Babb (1991) noted that in Tennesse by mid-November, *H.chrysoscelis* moved from their arboreal resting sites, apparently to their terrestrial hibernation sites, with one specimen found under 8cm of soil.

Ritke, Babb & Ritke (1990) noted differences in life history patterns between disjunct populations of *H.chrysoscelis*. Geographical variation was documented for male chorus activity, minimum size at sexual maturity and mean body size. Furthermore, after the treefrogs first emerged in March, individuals were not found in the trees on non-breeding nights. Ritke *et al.* (1990) assumed that, on these nights, the frogs were foraging on the ground.

Character displacement has been indicated for sympatric populations of diploid and tetraploid Gray treefrogs by Ptacek (1992) with regard to male calling sites. It is possible that character displacement may also occur in other aspects of their ecology, for example, Ralin (1968) also working with sympatric populations, found that *H.versicolor* consumed proportionately more terrestrial insects than *H.chrysoscelis*. Therefore, in areas of sympatry, it might be expected that the parasite fauna recovered from both host species may show interspecific variation. Ritke, Babb & Ritke (1991) documented strong breeding site specificity and little movement between sites; therefore, it is possible that the parasite fauna may vary between localities on a fine scale. McAlpine *et al.* (1992) found that *H.versicolor* typically bred at sites which were created by road construction or gravel

excavation. This preference will also influence the exposure of hosts to digenean infective stages, depending upon the age of the pond and its colonisation by molluscan intermediate hosts.

In studies over the past 50 years, 31 parasite species have been reported from *H.versicolor* compared to only 3 from *H.chrysoscelis* (Adamson, 1981; Baker, 1977, 1978; Baker & Adamson, 1977; Brooks, 1976; Campbell, 1968; Paul, 1938; Prudhoe & Bray, 1982; Reilly & Woo, 1982; Shannon, 1988; Woo & Bogart, 1984). These records are summarised in Table 8.1 and originate from a number of States in the U.S. and Canada.

In addition to the further documentation of the parasite fauna of both hosts, (particularly *H.chrysoscelis*), it was hoped to examine the potential influence of ploidy level on parasitic infection. In areas of sympatry it is possible to avoid gross ecological segregation, which may help determine whether any faunal variation between the species can be explained in terms of genetic or behaviourial differences.

8.3 Materials and Methods.

As described in Chapter 6, field collections were made of 182 specimens of the two host species (92 *H.chrysoscelis* and 90 *H.versicolor*). Those collected from sites 1, 2, 3, 7, 8, 9, 10 and 11 (full descriptions are recorded in Chapter 6) were dissected, within a few days of capture. The sample taken from site 12 was maintained in captivity for 9 days before necropsy. Frogs from sites 4, 5 and 6 were fixed in formalin the day after capture and transferred to 70% alcohol one week later.

The dissection procedure was as described in Chapter 3. All major organ systems were examined for possible parasitic infection, in particular, the eyes, nares, mouth, male vocal sac, lungs, stomach, intestine, rectum, urinary bladder and kidneys. Furthermore, a thorough search for encysted parasites was made. The epidermis was removed and all major muscle blocks teased apart. Although blood samples were taken from hosts collected at sites 1, 2, 3, 7, 8, 9, 10 and 11 to measure the packed cell volume (PCV), no smears were made to check for protozoan/filarial blood parasites. The parasites were fixed in a 10% formal saline solution, in the following manner: Monogenea and Digenea were fixed under 22x22mm coverslips, with sufficient pressure to display the hamulus and ventral sucker profile respectively. The single cestode was flattened under a second microscope slide. Nematodes were fixed in hot 70% alcohol. On return to London, monogeneans, digeneans and cestodes were stained, dehydrated and mounted *in toto* as described in Chapter 3. Nematodes were temporarily mounted in glycerol for identification. All parasites were identified to group and where

possible to genus and species.

8.4 Results.

The extent of the parasite fauna recovered from 92 *H.chrysoscelis* and 90 *H.versicolor* is recorded in Table 8.2. The prevalence and mean intensity of the four most common parasite species, from the sample as a whole, are summarised in Table 8.3. As described in Chapter 6, the three main sampling sites were: Phelps Co., Missouri; Boone Co., Missouri and Leon Co., Florida (sites 1, 2, & 6 respectively). The four most common parasites recovered from the treefrogs at these sites form the basis of the individual Mann-Whitney tests, documented in Table 8.4.

Representatives of the genus *Polystoma* were recovered from both sympatric populations (Missouri & Minnesota) and allopatric populations (Missouri, Texas, Florida & Louisiana). Prevalences ranged from 20.0% to 100% (Table 8.3), with burdens of 1-16 worms/host. Infection levels in Phelps Co., Missouri, the sympatric and syntopic site, were similar in the two host species (*H.chrysoscelis*: prev. = 29.4%; int. = 1.2 & r.d. = 0.35 and *H.versicolor*: prev. = 28.6%; int. = 1.8 & r.d. = 0.5). The distribution of the parasite population within the random host sample at site 2 was also comparable between the two host species (Fig.8.1). Although site 6 appears to have a different population structure (Fig.8.1), individual Mann-Whitney tests for each host species at sites 1, 2 and 6 indicate no significant differences (Table 8.4). As described in Chapter 6, it appears that a single species, *P.nearcticum* (Paul, 1935) Price, 1939 infects both

H.chrysoscelis and *H.versicolor* at a number of locations throughout their range. The new host record and five new locality records for *P.nearcticum* are fully described in Chapter 6.

Only one *H.chrysoscelis* was infected with *Clinostomum complanatum* Rudolphi, 1819: 31 metacercariae were recovered encysted in the mesenteries of a host from Phelps Co., Missouri. This does however, present a new host record.

Megalodiscus temperatus (Stafford, 1905) Harwood, 1932 was recovered from the intestine of the single female *H.chrysoscelis* from Louisiana. Specimens were also found in one *H.versicolor* from Wood Co., Texas (3 worms) and one *H.chrysoscelis* from Oklahoma (1 worm). This digenean has not previously been recorded from *H.versicolor* and therefore presents a new host record.

A single adult nematotaeniid cestode, *Cylindrotaenia americana* Jewell, 1916 was found in the intestine of the single *H.chrysoscelis* from Louisiana. Protocephalidea were found encysted in the tissue surrounding the small intestine of both host species at a wide variety of sites: Boone, Phelps and Barry Counties, Missouri; Kentucky; Florida and Oklahoma. Prevalences ranged from 3.7% to 33.3%. An interesting point is that no cysts were recovered from *H.versicolor* at Phelps Co. compared to the 17.6% prevalence in *H.chrysoscelis* (n = 3/17).

Cosmocercella haberi Steiner, 1924 was found in the intestinal and rectal lumen of both *H.chrysoscelis* and *H.versicolor.* This was the most common parasite

species recovered, with both host species infected at all sites, except for Maryland, Oklahoma and Nebraska. Prevalences ranged from 20.0% to 100%, with a range of 1-294 worms/host. Even in large host samples, such as that of *H.chrysoscelis* from Florida, the mean intensity of infection was 169.9 worms/host. Frequency distribution plots for the three major sampling sites indicate that *H.chrysoscelis* harbours higher population densities than *H.versicolor*, both in sympatry and allopatry (Fig.8.2). However, individual Mann-Whitney tests (Table 8.4) reveal that there were significant differences between sites but not host species. Once again, this represents a new host record (*H.chrysoscelis*) for this parasite and a number of new locality records for both host species.

Nematodes of the genus *Dorylaimus* were recovered from the sclera and adipose tissues of the eye from both host species. This nematode was found at all the sites sampled except for Maryland, Minnesota, Nebraska and Oklahoma. The majority recovered were larval stages, only 5.1% of the 2337 worms recovered were adults, however, it was not possible to identify this parasite group to species level. Prevalences ranged from 8.3% to 100%, at mean intensities of 4.0 to 131.0 (Table 8.3) and a range of 1-162 worms/host. Frequency distributions of *Dorylaimus sp.* at the Sites 1, 2 and 6 (Fig.8.3) suggest that there were differences between the populations of this nematode harboured by *H.chrysoscelis* and *H.versicolor* in allopatry and syntopy. This is confirmed by the individual Mann-Whitney tests (Table 8.4), which show significant differences above the 95% confidence level, between both hosts and sites. The infection of *H.chrysoscelis* provides a new host record and the study provides a number of new locality records for both host

species.

Third-stage larvae of the genus *Physaloptera* Rudolphi, 1819 were found firmly attached to the gastric mucosa of both *H.chrysoscelis* and *H.versicolor*. Prevalences ranged from 8.3% to 50.0%, at relatively low mean intensities of 1.0 to 10.5 and burdens of 1-51 worms/host. This nematode was not as widespread as *Cosmocercella haberi* or *Dorylaimus sp.*. The frequency distributions and statistical tests (Fig.8.4 & Table 8.4) indicate no significant differences between *H.chrysoscelis* and *H.versicolor* in allopatry or sympatry. This parasite has not previously been recorded from the diploid Gray treefrog.

The lung nematode *Rhabdias ranae* Walton, 1929 was found exclusively in *H.chrysoscelis* from Florida at low infection levels. Only 3 of the 27 specimens examined at this site harboured *R.ranae*, all were single burdens.

Encysted larval nematodes were rare with only one *H.versicolor* from Boone Co., Missouri infected and two *H.chrysoscelis*, one from Barry Co., Missouri and one from Ballard Co., Kentucky. The cysts were located in the connective tissue surrounding the intestine.

The endocommensal rectal protozoans *Balantidium sp.*, *Nyctotherus sp.* and *Opalina sp.* were widespread. Only the presence of these parasites was recorded and no distinction was made between *Balantidium sp.* and *Nyctotherus sp.* at dissection. The *Balantidium sp./Nyctotherus sp.* were absent from samples of

H.versicolor from Minnesota and *H.chrysoscelis* from Nebraska and Oklahoma. *Opalina sp.* was not recorded from the above sites nor from *H.versicolor* from Barry Co., Missouri.

A suite of host factors were recorded (snout-vent length, body weight, fat body and gonad weight). However, no significant correlations between host factors and infection with *C.haberi*, *Dorylaimus sp.*, *Physaloptera sp.* or *P.nearcticum* at the three largest sampling sites (1, 2 & 6) was found. In addition, the PCV, which may be depleted by the presence of blood feeding parasites, showed no correlation with infection. Weight values were only compared within samples, depending whether specimens were dissected fresh or preserved prior to dissection. Due to the sampling methods employed, very few females were collected (19/182) so no comparisons on the basis of host sex could be made.

8.5 Discussion.

The two previous parasitological surveys of Gray treefrogs both concerned *Hyla versicolor* alone. Campbell (1968) recorded 6 helminth species from 28 *H.versicolor* from Virginia, which included *Polystoma nearcticum*, *Cosmocercella haberi* and *Physaloptera ranae*. In addition, the nematodes *Cosmocercoides dukae* and *Oxysomatidium variabilis* were recovered from the intestinal lumen and *Foleyella americanum* from body cavities. Shannon (1988) added 6 new species records (*Haematoloechus sp.*, *Clinostomum marginatum*, diplostomatid metacercariae, *Dorylaimus sp.*, proteocephalid cestodes and porrorchid acanthocephalans) from 271 *H.versicolor* dissected from 2 sites in Missouri,

(including the Ashlands Reserve).

Detailed examination of the literature relating to the parasites of Gray treefrogs, reveals records of Monogenea (Campbell, 1968; Paul, 1938; Shannon, 1988), Digenea (Prudhoe & Bray, 1982; Shannon, 1988; Walton, 1964), Cestoda (Shannon, 1988), Nematoda (Adamson, 1981; Baker, 1977, 1978; Baker & Adamson, 1977; Campbell, 1968; Shannon, 1988), Acanthocephala (Shannon, 1988) and Protozoa (Campbell, 1968; Reilly & Woo, 1982; Shannon, 1988; Woo & Bogart, 1984).

Polystoma nearcticum recovered in this survey has been discussed at length in Chapter 6. However, in addition to the findings already presented, further analyses (individual Mann-Whitney tests) confirm that there were no significant differences between the populations recovered from either host species in syntopy or allopatry.

The digenean fauna in this study was restricted. It is surprising not to have recovered a larger number of *Clinostomum complanatum*, as this is a ubiquitous parasite of amphibians in the U.S.A. (see Chapter 5), however this may be related to host ecology (discussed below). No clinostomatids have been reported from Gray treefrogs prior to 1982 (Prudhoe & Bray, 1982). Shannon (1988) and Hausfater, Gerhardt & Klump (1990) first reported *C.marginatum* (syn *C.complanatum*) from *H.versicolor* at the Ashlands Reserve. Therefore, the discovery of *C.complanatum* in *Hyla chrysoscelis* does present the first record of infection by this parasite in the diploid Gray treefrog.

Megalodiscus temperatus was also rare. Prudhoe & Bray (1982) stated that *M.temperatus* has been reported from a number of amphibian species in Louisiana, Oklahoma and Texas, but not from Gray treefrogs. Sey (1991) documented records of *M.temperatus* from the United States, Canada, Mexico and Costa Rica with a broad host range (including *Hyla cinerea*, *H.chrysoscelis* and *H.crucifer*). Furthermore, Dyer (1991) reported *M.temperatus* from *H.crucifer* in Michigan, and McAllister *et al.* (1993) reported *M.temperatus* from *H.avivoca* in Arkansas. The sole record of this amphistome from Gray treefrogs comes from Brooks (1976) with the recovery of infected *H.chrysoscelis* in Nebraska.

Krull & Price (1932) found that the snail, *Helisoma trivolis* was a natural intermediate host of *M.temperatus*. However, Smith (1967) also noted that ancylid snails (*Ferrissia spp.*) were infected with larval *M.temperatus*. The cercariae prefer the pigmented areas of amphibian skin for encystment sites, rarely encysting on vegetation (Krull & Price, 1932). The cysts are viable for approximately one week, with hosts becoming infected after ingesting sloughed skin. Tadpoles, as young as 10 days post-hatch, may ingest cysts which have become detached and settled on the detritus at the bottom of the pond (Herber, 1938).

The adult nematotaeniid cestode, *Cylindrotaenia americana* Jewell, 1916, has been reported from 36 species of amphibians and reptiles, mainly in the nearctic (McAllister, 1991). Of these records, 7 are from hylids, including *Hyla arenicolor* and *H.squirella*. Subsequently, McAllister *et al.* (1993) have added *H.avivoca* to the list. Stumpf (1982a) supported the direct life-cycle reported for *C.americana*,

the attempted experimental infection of tadpoles and potential insect intermediate hosts failed. In the laboratory, infective stages may remain viable for 8 to 10 days in moist conditions (Stumpf, 1982b).

Although the protocephalid larval cestodes are transmitted by prey intake, with the small number recovered, there is little scope for analysis of habitat partitioning between hosts on the basis of diet. However, an interesting point is that no Protocephalidea were recovered from *H.versicolor* at Phelps Co. as opposed to the 17.6% prevalence in *H.chrysoscelis*; this may be due to sample size or possibly some degree of niche separation.

Previous studies have suggested that *Cosmocercella haberi* is an extremely common parasite of Gray treefrogs (Baker & Adamson, 1977; Shannon, 1988), and this has been confirmed in this study. *C.haberi* was first described from *Hyla carolinensis* by Steiner (1924) who concluded that it was 'improbable that this parasite..is any way harmful to its host'. Baker & Anderson (1977) redescribed *C.haberi* from *H.versicolor* in Ontario and proposed that this species infects Gray treefrogs throughout the United States. Although the life-cycle has not been fully characterised, female *C.haberi* are viviparous, producing large L1 larvae which Steiner (1924) suggested did not leave the host of their mother.

Other members of the genus Cosmocercidae have a direct mode of transmission with embryonated eggs released with host faeces. After hatching, larval nematodes inhabit the soil surface/leaf litter and may possibly enter a free-living phase. The

alternative path results in an infective larva penetrating the hosts skin and migrating to the alimentary tract. Experimental studies have also successfully demonstrated oral inoculation, in addition to percutaneous transmission (Baker, 1989). Baker (1989) concluded that 'given the food habits and biology of the hosts of the Cosmocercidae this transmission pattern may be widely distributed'.

Physalopterids have been recorded from *H.versicolor* by Walton (1931); however, these specimens were adult *Physaloptera ranae*, the only reported physalopterid to utilise amphibians as a definitive host in the U.S. It is probable that the treefrogs harbouring larval *Physaloptera sp.* recovered in this study, were acting as paratenic hosts, a common feature of this parasite group (Baker, 1989). Schmidt & Roberts (1989) reported that the physalopterid life-cycle involves an arthropod intermediate host, such as a cricket or cockroach, possible prey items for treefrogs, as well as the correct (definitive) host.

Dorylaimids are common free-living soil nematodes, with a few species parasitising plants (Thorne, 1961). Poinar (1983) estimated that one quarter of free-living nematodes were of the order Dorylaimida. Goodney (1963) stated that nearly all *Dorylaimus* were aquatic or from extremely moist habitats. Shannon (1988) first recorded dorylaimid infection of *Hyla versicolor.* The fact that larval and adult stages were recovered from the same habitat indicates that the life-cycle is being completed in the tissues of the eye. The majority of specimens recovered in this study were firmly attached to the inside of the eyelids and nictitating membrane, indicating that they may use their piercing stylets to feed on host

tissues. Infection most probably occurs during host hibernation in the soil or leaf litter, with infective stages entering the moist environment of the eye.

The genus *Rhabdias* Stiles & Hassall, 1905 has a cosmopolitan distribution with representatives occurring in a number of amphibian and reptilian families (Baker 1978). *Rhabdias ranae* has an indirect (heterogonic) life-cycle which may be free-living as well as parasitic. Baker (1979a) followed the parasitic life-cycle by experimental infection. Larvae penetrated the skin, initially moving subcutaneously, then through skeletal muscle into the body cavity, finally reaching the lungs. The adult worm lives as a protandric hermaphrodite and feeds on blood. Eggs hatch in the lung and L1 larvae pass out with the faeces. After moulting to the L3 stage, worms may infect the definitive host (alternatively a molluscan paratenic host) or take the free-living path. Fully embryonated eggs are produced as early as 9 days p.i. The different species of *Rhabdias* investigated were successfully cross-infected, leading Baker (1979a) to postulate that ecological factors and the reduced infectivity of larvae penetrating a heterospecific host resulted in their apparent host specificity.

Baker (1979b) suggested that there were one (or possibly two) generations of *R.ranae* each year, transmission reaching a maximum during the late summer/early autumn. Populations infesting *Rana sylvatica* were overdispersed with a maximum of 88 worms/host, the majority of infected frogs harbouring 1-5 worms. The longevity of infection was suggested to be the result of low host body temperature during hibernation.

The endocommensal *Opalina* Purkinje and Valentin, 1840 were not identifiable to species. Protozoans of this genus were first reported from *H.chrysoscelis* by Metcalf (1923). The identification of opalinids is problematic, due to the inadequate descriptions and taxonomy (see McAllister, 1987). The ciliate *Nyctotherus* Ehrenberg also infected the rectum, *N.cordiformis* was reported from *H.versicolor* by Walton (1947). However, no distinction was made between *Nyctotherus sp.* and *Balantidium sp.* Transmission is restricted, with infective stages released from the adult during their punctuated visits to waterbodies. Tadpoles become infected after ingesting the encysted protozoans.

A number of the parasite species recovered in this study were larval stages (*C.complanatum*, protocephalid cestodes, *Dorylaimus sp.*, *Physaloptera sp.* and encysted larval nematodes), utilising Gray treefrogs as intermediate or paratenic hosts. This suggests that the treefrogs may be common prey items for other vertebrates. Where the treefrog represents the definitive host, some of the parasite species were recovered in very small numbers (*M.temperatus*, *C.americana* and *R.ranae*), from which no significant conclusions regarding the effects of host ploidy or ecology can be drawn. Those species which are possibly transmitted by diet (protocephalid cestodes, *Physaloptera sp.* and encysted larval nematodes) were again typically found at low intensities. However, as noted above, those protocephalids recovered from Phelps Co., a sympatric and syntopic site, were encysted only in *H.chrysoscelis*, which may indicate a degree of niche separation in this region. Finally, *P.nearcticum* and *C.haberi* which were common and employ Gray treefrogs as definitive hosts, did not show significant differences in

distribution between the two host species in sympatry, although variation in populations by locality was found for the nematode (individual Mann-Whitney tests).

The somewhat restricted fauna (particularly the Digenea) may best be explained by the arboreal habitat of the host. Brandt (1936) and Campbell (1968) found that the more aquatic the amphibian host is, the greater its diversity of parasitic infection (particularly regarding digenean and blood protozoans). Therefore, the almost exclusively terrestrial/arboreal Gray treefrogs will have limited exposure to water-borne infective stages. This may be accentuated by the preference of Gray treefrogs for ponds arising from human activity (McAlpine *et al.*, 1992), as these may not contain suitable molluscan intermediate hosts to complete the life-cycle.

One would expect that with increasing host age, those parasites which have one opportunity to infect (*P.nearcticum*, rectal protozoans) to decline in prevalence and intensity and those which may have the possibility to transmit continuously (particularly *C.haberi* and *R.ranae*) to increase. Host snout-vent length and body weight (rough measures of age), did not reveal any correlation. By examining the age population structure of the host in more detail, possibly by skeletochronology, such patterns of infection may become apparent.

Where transmission may be punctuated (water or prey-borne infective stages) the distribution of parasites may be harder to predict. Patterns may be complex, for example, the frogs must re-hydrate at, as yet, undocumented intervals.

Furthermore, little is known about food preferences in different geographical locations, both in sympatry and allopatry. In this study, a number of the sampling sites were from sympatry, with both host species harbouring a virtually identical fauna. Ritke & Babb (1991) suggested that male *H.chrysoscelis* were non-territorial outside the breeding season which may allow overlap of habitat/foraging preferences of the diploid and tetraploid forms. However, although *Dorylaimus sp.* is non-specific, a significant difference was found between host species at Phelps Co. ($p < 0.05$), in addition to geographical differences. Conceivably this may indicate that *H.chrysoscelis* has some different hibernation and/or terrestrial habits than *H.versicolor* in this region. The typically higher prevalences and intensity of infection observed in Florida, may be due to the prevailing (warmer) climate and shorter winters.

Shannon (1988) documented moderate fluctuations in the infection levels of each parasite species at the Ashlands Reserve over a three year period. However, the prevalences and intensities in the present study were typically lower, which may be the result of heavy sampling of the host population by other research groups, lowering the median age of the treefrog population.

Without further information on the ecology and behaviourial traits of these cryptic hosts one can only speculate on specific aspects of parasite transmission processes and the resulting faunal distribution. However, from the data presented concerning specific parasites of hylids, no differences between *H.chrysoscelis* and *H.versicolor* on the basis of ploidy are evident. The significant differences in the

distribution of the non-specific *Dorylaimus sp.* may nevertheless indicate heterogeneity in ecological preference.

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A) - *H. versicolor*	Site of Infection.
Monogenea.	
Polystoma nearcticum (Paul 1935) Price, 1939	urinary bladder
Digenea.	
Haematoloechus sp.	lungs
Clinostomum marginatum Rudolphi 1819 (metacercariae)	general
Apharyngostrigea pipientis (metacercariae)	general
Gorgodera amplicava Looss, 1899 (metacercariae)	general
Cercaria amherstensis	general
Cercaria holthauseni	general
Cestoda.	
unidentified plerocercoids	intestine
Acanthocephala.	
cystocanth larvae of the subfamily Porrorchinae	general
Nematoda.	
Cosmocercella haberi Steiner 1924	colon
Cosmocercoides dukae Holl 1928	colon
Dorylaimus sp.	eye
Foleyella americana Walton 1929	mesenteries
Gryinicola batrachiensis Walton 1929 (tadpoles only)	colon
Larval nematodes	general
Oxysomatidium variabilis Harwood 1930	colon
Oswaldocruzia sp.	colon
Physaloptera ranae Walton 1931	stomach
Rhabdias ranae Walton 1929	lungs
Protozoa.	
Balantidium sp.	colon
Hexamita intestinalis Dujardin 1841	colon
Nyctotherus cordiformes Ehrenberg 1838	colon
Opalina hylaxena Metcalf 1923	colon
O. niangulata Metcalf 1923	colon
Tritrichomonas augusta Alexieff 1911	colon
Trypanosoma rotatorium Mayer 1843	blood
T. andersoni Reilly & Woo 1982	blood
T. grylli Nigrelli 1945	blood
T. pipientis Diamond 1950	blood
T. ranarum Danilewsky 1885	blood
B) - *Hyla chrysoscelis.*	
Digenea.	
Haematoloechus complexus (Seely, 1906) Krull, 1933	lungs
Megalodiscus temperatus (Stafford, 1905) Harwood 1932	rectum
Cestoda.	
Cylindrotaenia americana Jewell, 1916	intestine

Table 8.1. The parasitic fauna of the North American Gray treefrogs; **A** - *Hyla versicolor* and **B** - *H. chrysoscelis*. Complied from Walton, 1964; Campbell, 1968; Brooks, 1976; Prudhoe & Bray, 1982; Shannon, 1988.

	Hyla chrysoscelis	*Hyla versicolor*
Monogenea	*Polystoma nearcticum*	*Polystoma nearcticum*
Digenea	*Megalodiscus temperatus*	*Megalodiscus temperatus*
	Clinostomum complanatum	
Cestoda	*Cylindrotaenia americana*	
	Protocephalidea	Protocephalidea
Nematoda	*Dorylaimus sp.*	*Dorylaimus sp.*
	Cosmocercella haberi	*Cosmocercella haberi*
	Physaloptera sp.	*Physaloptera sp.*
	Rhabdias ranae	
	Larval cysts	Larval cysts
Protozoa	*Balantidium sp./ Nyctotherus sp.*	*Balantidium sp./ Nyctotherus sp.*
	Opalina sp.	*Opalina sp.*

Table 8.2 The parasite fauna of the North American Gray treefrogs, *Hyla chrysoscelis* (n = 92) and *Hyla versicolor* (n = 90) recovered from 12 collection sites in the U.S.A.

Site No.	Host Species	n	*P.nearcticum* prev.	int.	*C.haberi* prev.	int.	*Dorylaimus sp.* prev.	int.	*Physaloptera sp.* prev.	int.
1	*H.v.*	60	26.1*	1.4	40.0	74.2	45.0	19.5	21.7	9.1
2	*H.c.*	17	29.4	1.2	82.4	77.8	64.7	56.6	23.5	4.0
2	*H.v.*	14	28.6	1.8	64.3	47.1	35.7	4.0	28.6	7.3
3	*H.c.*	13			33.3	18.5	8.3	11.0	8.3	1.0
3	*H.v.*	3			33.3	129.0	33.3	131.0		
4	*H.c.*	4	25.0	1.0	100.0	79.0	50.0	42.5	50.0	10.5
5	*H.c.*	4			50.0	140.5	50.0	56.5	25.0	1.0
5	*H.v.*	5			80.0	87.0	40.0	30.5		
6	*H.c.*	27	33.0	4.9	74.1	169.9	62.9	43.0	11.1	1.7
7	*H.c.*	5			20.0	14.0				
7	*H.v.*	5	20.0	1.0	20.0	9.0				
8	*H.c.*	3			33.3	2.0				
8	*H.v.*	3							33.3	2.0
9	*H.c.*	7			57.1	37.3	14.3	7.0	28.6	2.0
10	*H.c.*	1	100.0	5.0	100.0	42.0	100.0	43.0		
11	*H.c.*	9							22.2	8.0
12	*H.c.*	2								

Table 8.3 Infection levels of the four most common parasites recovered from 182 Gray treefrogs at 12 sites in the U.S.A. (Abbreviations: prev. = prevalence; int. = mean intensity; *H.c.* = *Hyla chrysoscelis*; *H.v.* = *Hyla versicolor* and * = sample size (n) of 46).

i) - *Polystoma nearcticum.*

	Site 1 *H.v.* (*a)	Site 2 *H.c.*	Site 2 *H.v.*
Site 2 *H.c.*	0.9014	-	-
Site 2 *H.v.*	0.8271	0.9367	-
Site 6 *H.c.*	0.3789	0.5629	0.6402

ii) - *Cosmocercella haberi.*

	Site 1 *H.v.*	Site 2 *H.c.*	Site 2 *H.v.*
Site 2 *H.c.*	0.0120*	-	-
Site 2 *H.v.*	0.3697	0.2185	-
Site 6 *H.c.*	0.0017*	0.8002	0.9900

iii) - *Dorylaimus sp.*

	Site 1 *H.v.*	Site 2 *H.c.*	Site 2 *H.v.*
Site 2 *H.c.*	0.0279*	-	-
Site 2 *H.v.*	0.4315	0.0262*	-
Site 6 *H.c.*	0.0112*	0.7540	0.0128*

iv) - *Physaloptera sp.*

	Site 1 *H.v.*	Site 2 *H.c.*	Site 2 *H.v.*
Site 2 *H.c.*	0.8974	-	-
Site 2 *H.v.*	0.6340	0.7209	-
Site 6 *H.c.*	0.4116	0.4549	0.3025

Table 8.4 Individual Mann-Whitney tests for each of the four most common parasite species from the three major sampling sites. (Abbreviations: * = significant at or above the 95% confidence interval; *a for Site 1, n = 46, otherwise all sample sizes as stated in the Method).

8.7 Legends.

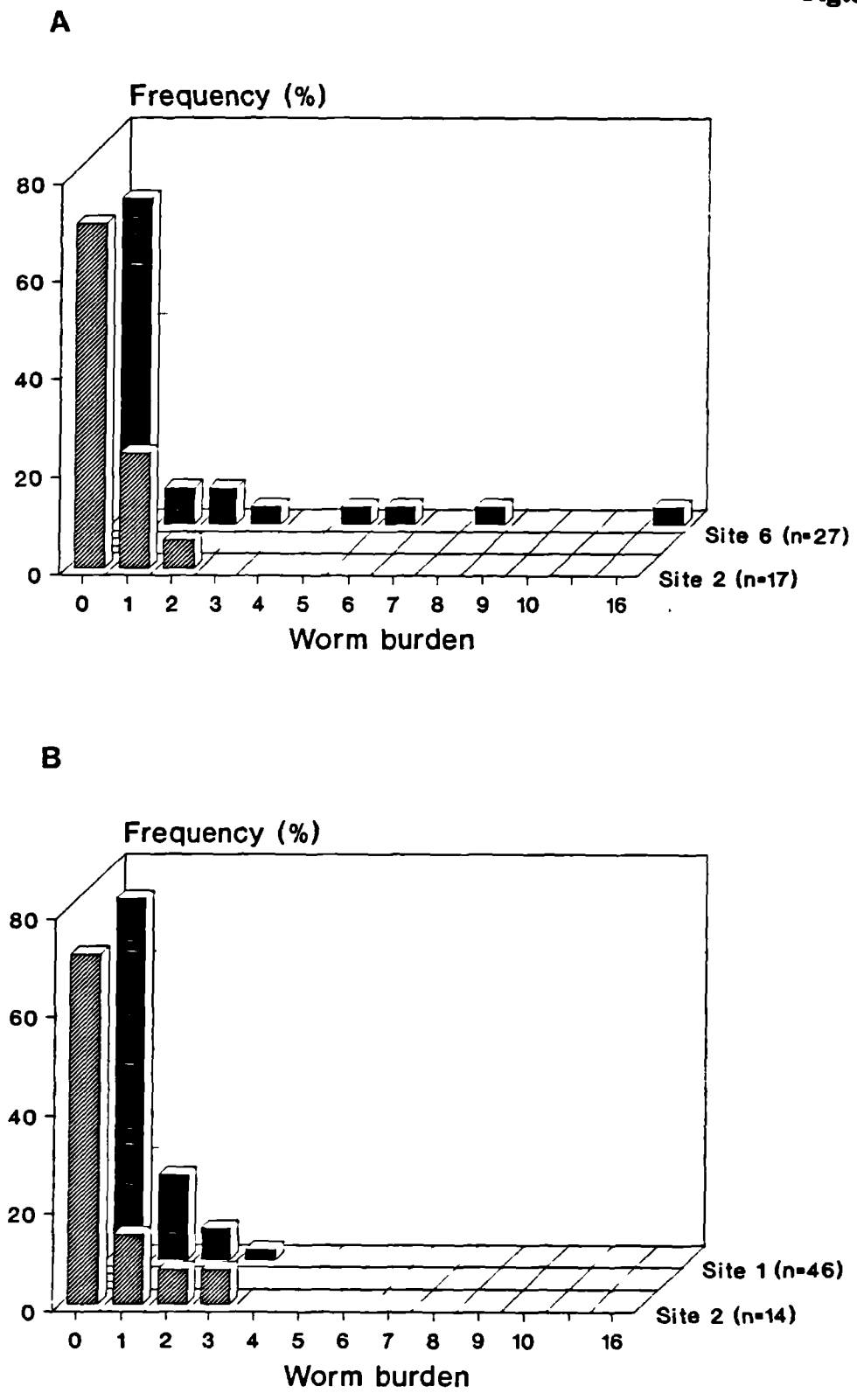
Fig.8.1 Frequency distribution of *Polystoma nearcticum* in A) *Hyla chrysoscelis* and B) *H.versicolor* from sites 1, 2 & 6. (Exact localities indicated in the text).

Fig.8.2 Frequency distribution of *Cosmocercella haberi* in A) *Hyla chrysoscelis* and B) *H.versicolor* from sites 1, 2 & 6. (Exact localities indicated in the text).

Fig.8.3 Frequency distribution of *Dorylaimus sp.* in A) *Hyla chrysoscelis* and B) *H.versicolor* from sites 1, 2 & 6. (Exact localities indicated in the text).

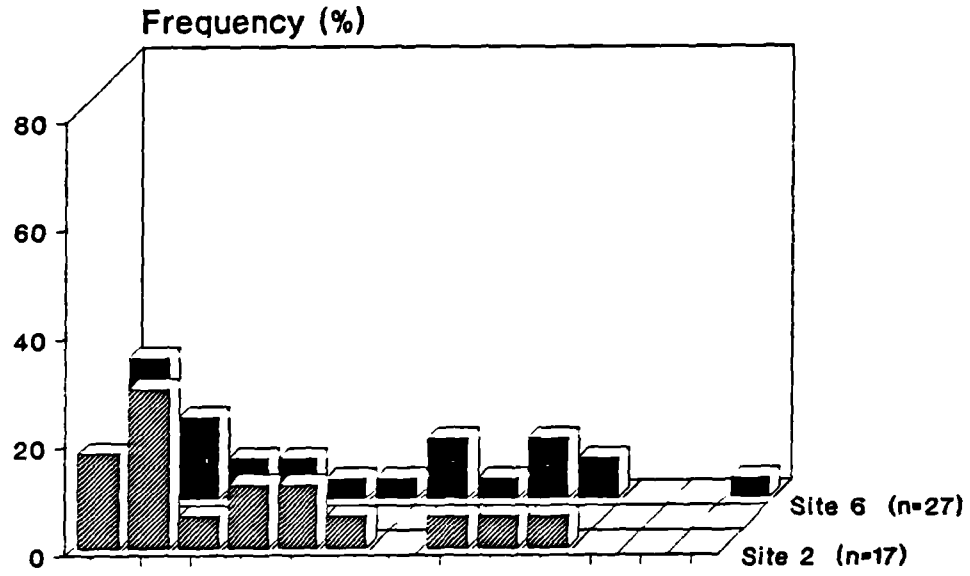
Fig.8.4 Frequency distribution of *Physaloptera sp.* in A) *Hyla chrysoscelis* and B) *H.versicolor* from sites 1, 2 & 6. (Exact localities indicated in the text).

Fig.8.1

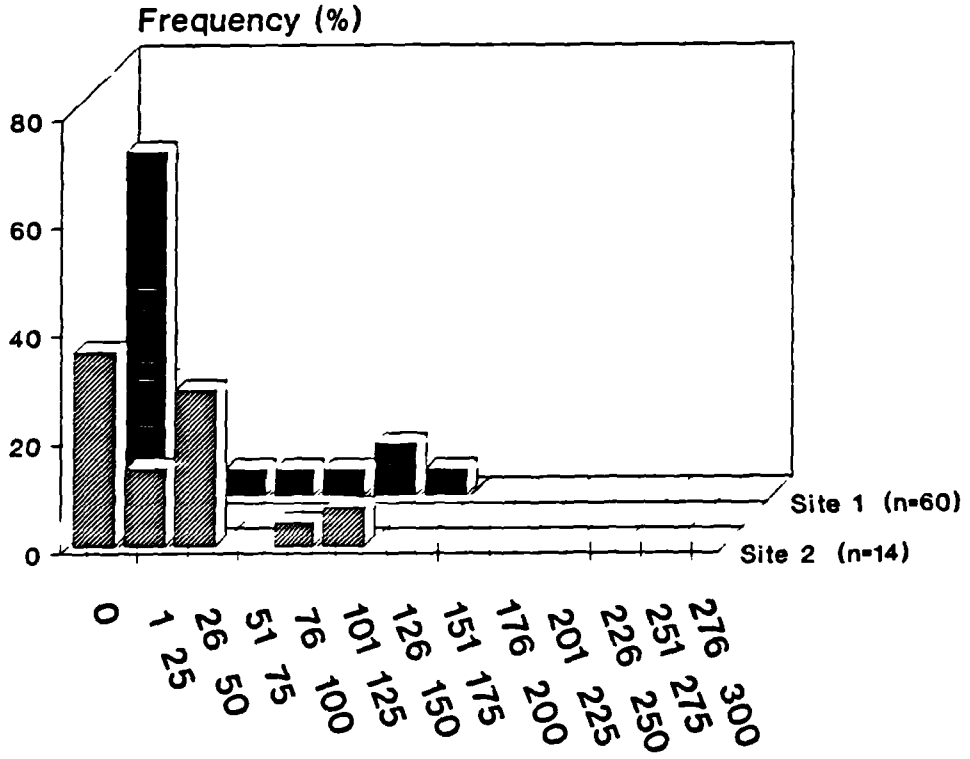

A
Frequency (%)
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 16
Worm burden
Site 6 (n=27)
Site 2 (n=17)
B
Frequency (%)
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 16
Worm burden
Site 1 (n=46)
Site 2 (n=14)

A

Fig.8.2

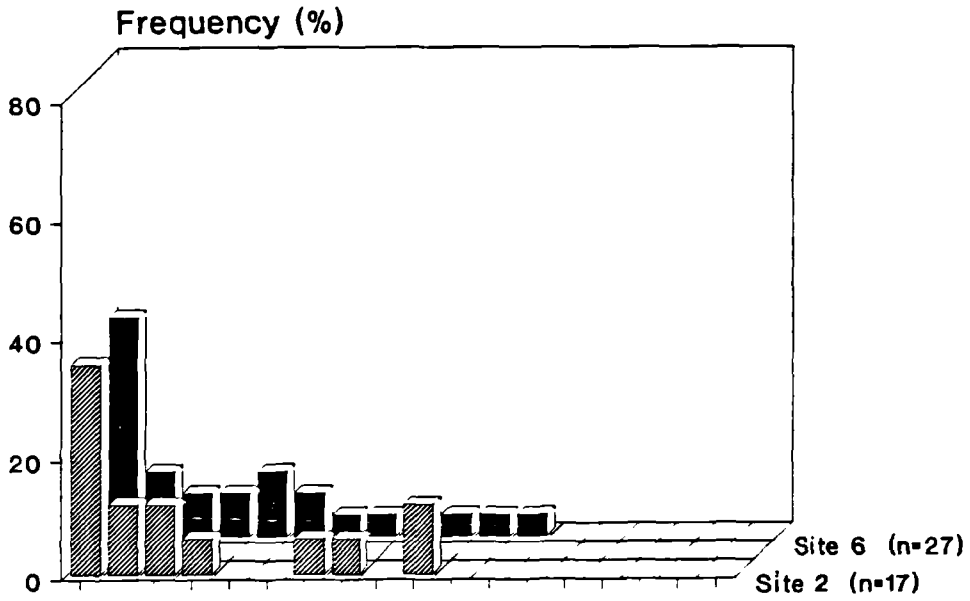

Frequency (%)
80
60
40
20
0
Site 6 (n=27)
Site 2 (n=17)

B


Frequency (%)
80
60
40
20
0
Site 1 (n=60)
Site 2 (n=14)
0
1 25
26 50
51 75
76 100
101 125
126 150
151 175
176 200
201 225
226 250
251 275
276 300
Worm burden

A

Fig.8.3


Frequency (%)
80
60
40
20
0
Site 6 (n=27)
Site 2 (n=17)

B

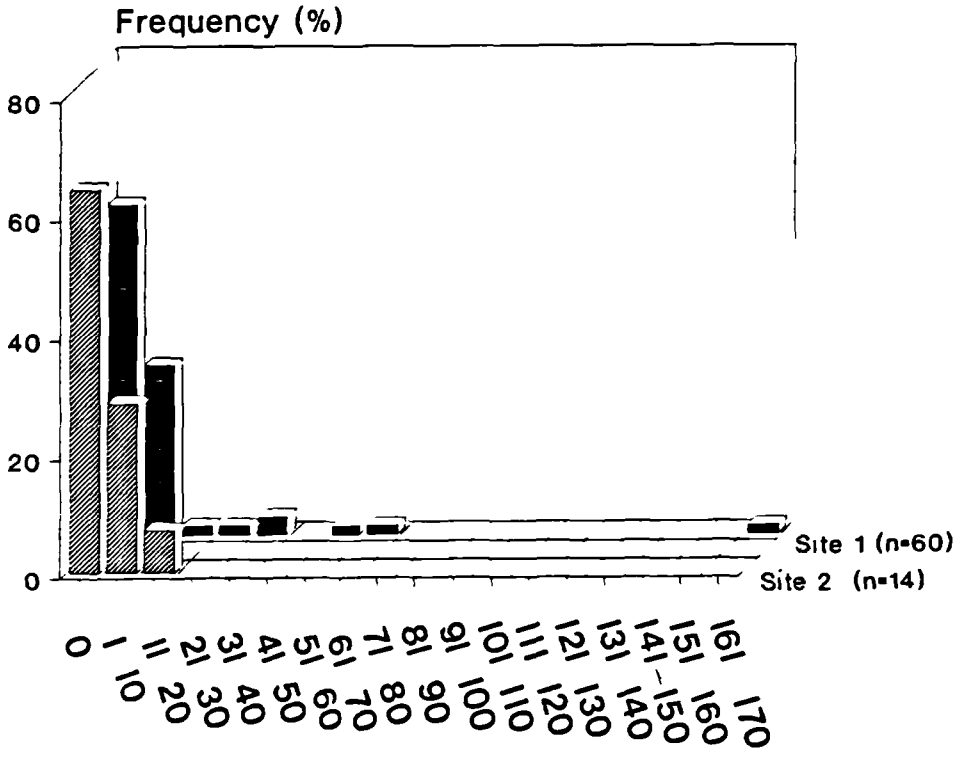
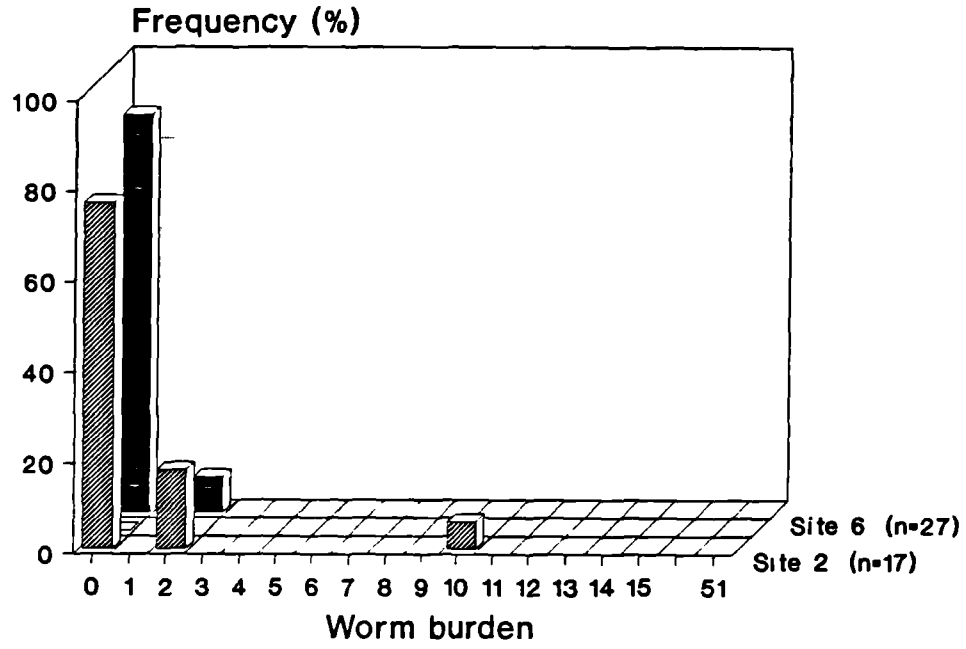
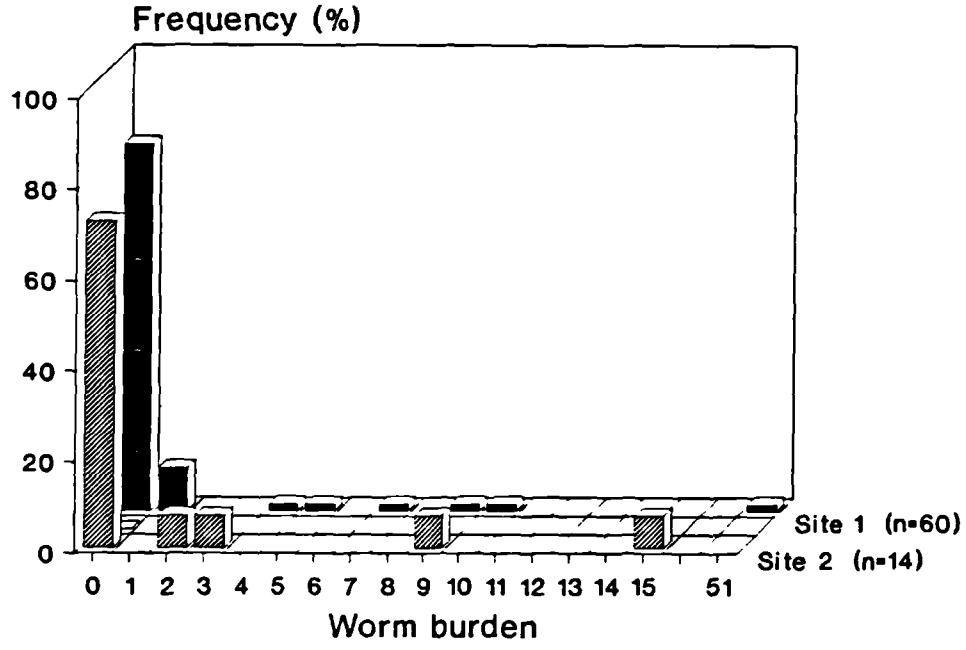

Frequency (%)
80
60
40
20
0
Site 1 (n=60)
Site 2 (n=14)
0 - 10
11 - 20
21 - 30
31 - 40
41 - 50
51 - 60
61 - 70
71 - 80
81 - 90
91 - 100
101 - 110
111 - 120
121 - 130
131 - 140
141 - 150
151 - 160
161 - 170
Worm burden

Fig.8.4

A


Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 51
Worm burden
Site 6 (n=27)
Site 2 (n=17)

B


Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 51
Worm burden
Site 1 (n=60)
Site 2 (n=14)

Chapter 9.

The distribution of infection in a controlled environment: the experimental infection of larval *Hyla versicolor* (Anura:Hylidae) with *Polystoma nearcticum* (Paul, 1935) Price, 1939 (Monogenea:Polystomatidae).

9.1 Abstract.

Tadpoles of the Gray treefrog, *Hyla versicolor* were exposed to the oncomiracidia of the polystomatid monogenean *Polystoma nearcticum*, under standard conditions with controlled variables of search area, host and oncomiracidial density. With increasing oncomiracidial density, the prevalence of infection rose rapidly in an exponential form, the majority of hosts becoming infected at densities of 0.22 parasites/ml and above. No correlation was found between the dimensions of the host (nose to tail-tip length or body weight) and the proportion of successful oncomiracidia/ml. Individual Mann-Whitney tests suggested that the spatial dimensions of the infection arena were not a significant factor in invasion success. For pooled samples of single tadpole exposures, a significant correlation was found between the mean worm burden/host and the initial oncomiracidial density (R^2 = 55.1%, F = 26.99, $p < 0.0001$). This trend was also found for tadpoles exposed in pairs (R^2 = 63.0%, F = 23.80, $p < 0.0001$), however, for groups of five hosts, the data fell just outside of the 95% confidence limit (R^2 = 60.7%, F = 6.17, p = 0.068 NS). Regression line gradients clearly indicate that with increasing host density, a greater number of parasites were recovered, however, the increases were disproportionate. In order to test whether size-related variation of hosts affected the likelihood of invasion, an index was developed for tadpoles exposed in pairs. The index revealed that host size may influence invasion success, particularly at low parasite densities. To examine the distribution of parasites, a second index was calculated for tadpoles exposed in pairs. At all parasite densities, the majority of parasites were found to be distributed in a manner tending towards random or underdispersed. In experiments

where tadpoles were exposed in groups of five, at all oncomiracidial densities, the variance/mean ratio (s^2/x) exhibited no clear pattern of parasite distribution. The results are discussed in relation to theoretical predictions and previous experimental investigations.

9.2 Introduction.

In the first quantitative assessment of parasite distribution within host populations, Crofton (1971) concluded that parasites were typically overdispersed, generally fitting a negative binomial distribution. Overdispersed distributions have been recorded in a number of parasitic groups, including the Monogenea (Frankland, 1955; Tinsley, 1993), Digenea (Pennycuick, 1971), Cestoda (Anderson, 1974a; Pennycuick, 1971; Wassom, Guss & Grundmann 1973; Wassom *et al.*, 1986), Nematoda (Northam & Rocha, 1958; Schmid & Robinson, 1972), Acanthocephala (Crofton, 1971; Pennycuick, 1971) and Arthropoda (Boxhall, 1974; Cole, 1949; Randolph, 1975). Furthermore, the limited number of random or underdispersed (homogeneous) distributions recorded in the literature were postulated by Anderson & May (1978a) to be the result of examining laboratory populations (Anderson, Whitfield & Mills, 1977; Northam & Rocha, 1958), specific age classes (Anderson, 1974b) or horizontal survey techniques (Stromberg & Crites, 1974).

Overdispersed distributions are those in which the variance is greater than the mean, with the minority of hosts harbouring the majority of parasites. The causal factors in producing aggregated distributions may act alone or in combination.

Contributory factors include the viability, spatial aggregation and behaviour of infective stages, in addition to the potential for direct reproduction within the host. Host-mediated factors include their behaviour, diet, age and immunological capabilities. Mathematical models and their relation to field data have provided a basis for testing the interactions within host-parasite systems (see Anderson & Gordon, 1982, Anderson & May 1978a, b; Crofton, 1971). Anderson & May (1978a) concluded that overdispersion will tend to produce stability, however, destabilising processes may include parasite-induced reduction in host reproductive potential, parasite reproduction within the host, and time delays in parasite reproduction and transmission (Anderson & May, 1978b). McCallum (1990) proposed that, theoretically, 'short-term' factors (e.g. encounter rate with patchily distributed infective stages) may have the more significant effect upon parasite distribution than 'long-term' factors (e.g. innate resistance).

Experimental studies have addressed specific questions regarding the overdispersion of parasites. Anderson, Whitfield & Dobson (1978) recorded that, under standard conditions, the numbers of successful invasions of *Tranversotrema patialense* were directly proportional to the initial cercarial density. However on a theoretical basis, Anderson (1978) noted that an upper extreme may be reached as an individual host of a particular size may only support a finite number of invading parasites within the spatial confines of its body. In addition, Anderson *et al.* (1978) found that the distribution of infection shifted from a random pattern to overdispersion as either cercarial density or exposure time was increased. This finding is reflected in nature, as Kennedy (1981) reported that following the

introduction of the digenean *Tylodelphys podicipina* into a lake system, infection levels were low and distribution was random during the initial stages of establishment. However, as parasite numbers increased overdispersion became distinct. The longevity of digenean miracidia was examined by Anderson *et al.* (1982) who documented that the death rate of oncomiracidia exhibited an exponential reduction with age. Infectivity also declined over time, although the survival and infectivity temperature optima were separated by 10^0C. Where a parasite species is transmitted by the ingestion of an intermediate host, Keymer & Anderson (1979) found that the mean worm burden rose to a plateau, with the nutritional status of the host population governing the level of the plateau.

Predisposition to infection has been demonstrated by Wassom *et al.* (1973, 1974, 1986) and Munger *et al.* (1989) for the cestode *Hymenolepis citelli* infecting two congeneric murine hosts. Wassom *et al.* (1973, 1974, 1986) considered the possession of a single dominant resistance gene by approximately 75% of the population and the spatial heterogeneity of infective stages to be the two major contributory factors in producing the overdispersed distribution of *H.citelli.* Inherent differences in susceptibility between the two host species was suggested to be related to the duration of evolutionary association between parasite and host (Munger *et al.*, 1989). Predisposition to infection has been proposed for mice infected by the nematodes *Heligosomoides polygyrus* and *Aspicularis tetraptera* (see Scott, 1988) and confirmed for the latter by Moulia *et al.* (1993), plus Coustau *et al.* (1991), mussels and for guppies infected by the viviparous monogenean, *Gyrodactylus bullatarudis* (see Scott, 1985). Furthermore, the

significant host mortality induced by *G.bullatarudis* was deemed to be an important factor in the regulation of the host population (Scott, 1985).

It is evident that the natural population dynamics of host-parasite relationships are produced by a combination of complex interactions. The distribution of infection within a controlled environment may separate the principal factors which produce overdispersed distributions. Therefore, the primary objectives of this study were to assess the transmission dynamics of the *P.nearcticum-H.versicolor* system in comparison to other host-parasite systems and then to address the distribution of infective stages if they are presented with a number of hosts in the same infection arena.

9.3 Materials and Methods.

Uninfected *H.versicolor* tadpoles were raised from 12 egg masses collected on the nights of 15/16th May, 1992 from the Ashlands Reserve, Boone Co., Missouri. The maintenance regime was as described in Chapter 7. A total of 595 tadpoles (aged 1-2 weeks post-hatch, p.h.) were exposed to oncomiracidia in the following combinations.

1.) Single tadpoles were exposed to 1, 2, 5 and 10 oncomiracidia in 5 or 8.5cm diameter petri dishes containing 5-45ml of aged water (refer to Table 9.1).

2.) Pairs of tadpoles were exposed to 2, 4, 10 and 20 oncomiracidia in a 8.5cm petri dish or 9.5cm glass dish containing 30-115ml of aged water (refer to Table

9.2A).

3.) Groups of five tadpoles were exposed to 5 and 10 oncomiracidia in a 9.5cm glass dish or 7x14cm plastic aquaria containing 70-120ml of aged water (refer to Table 9.2B).

All the containers were soaked in aged water for a minimum of 6 hours prior to use. These were then filled with the appropriate volume of water, measured with a calibrated pipette. Recently hatched oncomiracidia (less than 1h old) were taken up into a pasteur pipette and released into the centre of the infection dish with the minimum current. Only those oncomiracidia which swam vigorously were used for infection procedures. All oncomiracidia were counted under a binocular microscope as they entered the infection arena. After a 10 minute equilibrium period, tadpoles were placed in the dishes and left for 36h. The water temperature was maintained at 20.6+_0.8°C with thermostatically controlled air-conditioning units.

Single tadpole exposures were dissected in the laboratory at the University of Missouri, however, multiple exposures were fixed in 70% alcohol, with each tadpole stored in a separate eppendorf tube and dissected after return to Q.M.W. The tadpoles had any excess moisture removed on tissue paper and weighed on a top-pan balance (to the nearest 0.001g for single exposures, 0.005g for multiple exposures). The nose to tail-tip length was also recorded (to nearest 0.1mm & 0.5mm respectively). The dissection procedure was as described in Chapter 7,

with the number of worms and their sites of attachment recorded. For the fixed specimens, the eppendorf tubes were rinsed twice with 70% alcohol and the washings were examined for any detached worms, however, no worms were ever recovered free in the preservative.

9.4 Results.

9.4.1 Single tadpole exposures.

Although not quantified, the tadpoles initially moved around the infection arena and then settled. They remained motionless for the majority of the exposure period, unless disturbed by vibration, shadow or contact with another tadpole. As the infection dishes were placed away from disturbance, most tadpoles remained still, with only occasional, irregular movements.

At host dissection 74.2% of worms harboured blood in their guts. The prevalence of infection rose rapidly with increasing oncomiracidial density, following an exponential form, with the majority of hosts becoming infected at densities of 0.22 oncomiracidia/ml and above (Fig.9.1). With the exception of a single point (5cm petri dish, 0.4 oncomiracidia/ml, 60% prevalence), the prevalences for both types of infection arena fell within the same broad curve. By regression analysis, no correlation was found between the dimensions of the host (nose to tail-tip length or body weight) and the proportion of successful oncomiracidia/ml (Table 9.3):

1) host length v density, R^2 = 1.7%, F = 1.75, p = 0.189 (5cm petri dish).
2) host weight v density, R^2 = 1.9%, F = 1.99, p = 0.161 (5cm petri dish).
3) host length v density, R^2 = 2.2%, F = 2.65, p = 0.106 (8.5cm petri dish).
4) host weight v density, R^2 = 2.1%, F = 2.54, p = 0.114 (8.5cm petri dish).

Table 9.3 Regression analysis of host dimensions and the proportion of successful oncomiracidia/ml.

To assess directly the effect of area on the success of invasion, data from single tadpoles which were exposed to oncomiracidia in 15ml of water in both 5 and 8.5cm petri dishes were compared by individual Mann-Whitney tests. The tests revealed no significant differences between the two container types, except for doses of 2 oncomiracidia/ml.

8.5cm petri dish

Oncomiracidial dose	1	2	5	10
5cm petri dish	0.2057	0.0025*	0.7558	0.9097

Table 9.4 Individual Mann-Whitney tests of invasion success in relation to the infection arena (* : significant at or above the 95% confidence interval).

For pooled samples from both types of infection arena, a significant correlation was found between the mean worm burden/host and the initial oncomiracidial density (Fig.9.2, R^2 = 55.1%, F = 26.99, $p < 0.0001$). Furthermore, variation in mean worm burden increased as exposure density rose.

9.4.2 Multiple tadpole exposures.

A significant correlation was also found between the mean worm burden/host and the initial oncomiracidial density for tadpoles exposed in pairs (Fig.9.3A, R^2 = 63.0%, F = 23.80, $p < 0.0001$) and for groups of five hosts (the smallest sample), although a positive trend was noted, the data fell just outside of the 95% confidence limit (Fig.9.3B, R^2 = 60.7% F = 6.17, p = 0.068 NS). For pairs, as with single tadpole exposures, the variation in mean worm burden clearly rises with increasing oncomiracidial density. As described above, samples were pooled and both types of infection arena were incorporated into the analysis. The gradients of the regression lines calculated for each host density clearly indicate that, with increasing host density, a greater number of parasites were recovered at each oncomiracidial density (Fig.9.4). However, the increases were not proportional, with a two-fold increase in host density resulting in a 6-fold increase in parasite recovery and a 5-fold increase in density produces nearly a 10-fold increase in recovery.

For single tadpole exposures, no correlation was found between invasion success and host dimensions (Table 9.3), however, when the oncomiracidia were confronted with two potential hosts of different size, a preference may have been exhibited. In order to test whether dimorphic variation of hosts affected the likelihood of invasion, an index was developed for tadpoles exposed in pairs. Criteria were based on a number of qualifying factors. Firstly, that within each pair of hosts at least one successful invasion had occurred, the relevant proportion of total invasions was then allocated to each tadpole. To produce host size criteria,

regression analysis of the nose to tail-tip length and body weight of a sample of 104 tadpoles was undertaken. A highly significant correlation was found (Fig.9.5, $R^2 = 97.1\%$, $F = 3446.55$, $p < 0.0001$). The definition of the larger tadpole of the pair was based primarily on length, but if lengths were equivalent, the tadpole of higher body weight was selected. From these data a 'host size-invasion success' index was formulated as follows:

Index = -0.5 + mean proportion of the total successful invasions recovered from the larger host.

Therefore, values may range from the extreme of -0.5 (all oncomiracidia established upon the smaller host) to +0.5 (all oncomiracidia established upon the larger host), with an even distribution between both hosts producing a score of 0. The mean index value was plotted for each oncomiracidial density (a significant influence on mean worm burden for single and multiple exposures, Fig.9.6). At lower oncomiracidial densities (< 0.2 oncomiracidia/ml) there was a trend for the larger tadpole of the pair to be infected in preference to the smaller host. Below 0.14 oncomiracidia/ml, all mean values are at or above zero. Above 0.2 parasites/ml the trend reverses, with the majority of mean values falling within the preference range of the smaller tadpole. However, it should be noted there was considerable variation at all densities, the greatest variation occurring in those exposures involving 2 and 4 oncomiracidia.

To examine whether the invading oncomiracidia distributed themselves in a underdispersed, random or overdispersed manner, two analyses were undertaken. For tadpoles exposed in pairs a 'distribution index' was produced as follows:

$$\text{Index} = \frac{\text{difference in worm burden between hosts}}{\text{initial exposure dose of oncomiracidia}}$$

. Once again, only successful exposures were considered, the results are displayed in Fig.9.7. Therefore, extreme values of 0 and 1 indicate underdispersed and overdispersed distributions respectively, with a value of 0.5 indicating a random distribution. It is evident that at all densities the mean values fall at or below 0.5, with the majority of parasites distributed in a manner tending towards an underdispersed distribution. Once again the greatest variation occurred in those exposures involving 2 and 4 oncomiracidia.

In experiments where tadpoles were exposed in groups of five, for each replicate the variance/mean ratio (s^2/x) was calculated and plotted against oncomiracidial density (Fig.9.8). Values over 1 indicate an overdispersed distribution, approximately 1 indicates a random distribution and below 1 an underdispersed distribution. At all oncomiracidial densities, the variance/mean ratios were highly variable, exhibiting no clear pattern of distribution.

9.5 Discussion.

By the experimental investigation of host-parasite systems it is possible to control or moderate a number of the factors which influence the distribution of parasites within a host population. With regard to the factors stated in the introduction, it should be noted that the roles of host diet and direct parasite reproduction were not applicable to this particular system. A further four parameters were moderated; the viability and spatial aggregation of oncomiracidia, host age and, potentially, immunity.

Anderson *et al.* (1982) recorded that the death rate of miracidia declined exponentially with age, consequently the viability of oncomiracidia was based on strict criteria of utilising only those individuals less than 1h p.h. which swam vigorously. Furthermore, as stated in Chapter 7, the life-span of *P.nearcticum* larvae may approach 24h, thus the oncomiracidia were allowed sufficient time in which to locate a host. Spatial aggregation will always remain a possibility when infective stages are mobile, however, oncomiracidia were released into a simple arena in a standardised manner. Therefore, oncomiracidial distribution would have been dependent upon their swimming patterns. It is interesting to note that, for infective stages transmitted by ingestion, Keymer & Anderson (1979) found that the pattern of infection remained overdispersed even when infective stages were arranged in a uniform manner. However, the degree of overdispersion became accentuated as the spatial patterns of infective stages were changed from under-dispersed to overdispersed. In the present study, tadpole age was restricted to 1-2 weeks p.h. as it has been suggested that examining specific age groups may

produce misleading results (Anderson & May, 1978a), however, Pennycuik (1971) documented that three parasite species infecting adult *Gasterosteus aculeatus* remained overdispersed with regard to both host sex and age class.

The possibility of acquired immunity was controlled by utilising naive hosts, however, after only 36h over 74% of worms had ingested their first blood meal, which may have allowed the tadpoles an opportunity to respond to infection via innate resistance mechanisms. Following the discovery of a gene for resistance in the mouse *Peromyscus*, Wassom *et al.* (1986) emphasised that this simply provided another contributory factor to the overdispersion of the cestode *H.citelli* and not a full explanation. As described in Chapter 7, after establishment, members of the genus *Polystoma* may produce dimorphic larval stages. The change from the 'neotenic' to 'bladder-destined' form is dependent on the age of the tadpole when first encounted (Gallien, 1934; Murith, 1981a), so by using recently hatched tadpoles it was assumed that all worms would take the neotenic path. Both larval morphs feed on blood (Williams, 1960), although it has been suggested that they may be antigenically different (Murith, 1981b). Kok (1990) documented the steady decline of neotenic *P.umthakathi* suprapopulations, although accidental detachment was suggested as the principal cause of loss. This area of polystomatid biology remains unclear and the criteria employed in the present study will have reduced the influence of immunity to a minimum.

The density of oncomiracidia and tadpoles were controlled as they are, in conjunction with rates of contact and the survival of infective stages, principal

factors influencing direct transmission by an infective agent (Anderson, 1978; Anderson *et al.*, 1978; Keymer, 1982). Clearly, many of the factors are inter-related, for example, the rates of encounter between infective stages and potential hosts are dependant upon their respective densities and spatial distribution (Anderson, 1978; Crofton, 1971). In the present study, the single tadpole exposures displayed that, in this particular host-parasite system, the prevalence of infection may increase exponentially, a pattern also described by Keymer (1982) for *Hymenolepis diminuta*. Theory predicts that there is a linear relationship between the mean number of parasites per host and the initial density of infective stages (Anderson, 1982). In addition to the present study, other experimental investigations have confirmed this relationship (Anderson *et al.*, 1977, 1978; Grove & Warren, 1976; Lie, Heyneman & Kostanian, 1975). Moreover, Anderson *et al.* (1978) found that the distribution of infection shifted from a random pattern to an overdispersed form as cercarial density was increased, although a similar correlation was not found in this study. It is also interesting to note that, with increasing host density, there was a disproportionate increase in the numbers of parasites recovered and that the size of tadpoles may influence invasion success, particularly in a confined infection arena. The role of spatial influences on infection remain unclear, and although a significant difference was found between dishes at one parasite density, this was most probably an aberrant result.

The oncomiracidia of monogeneans are typically short-lived, a maximum of 4-6h for *Discocotyle*, 10h for *Diplozoon*, 17h for *Protopolystoma*, 30h for *Entobdella*, 32h for *Oculotrema*, reaching 48h for *Pseudodiplorchis* (all at their respective temperature optima - Kennedy, 1976; Tinsley & Earle, 1983; Tinsley & Owen, 1975) which leaves a finite time for the location of a suitable host. This may be achieved by the use of swimming patterns, chemoreception, photoreceptors and hatching rhythms (Llewellyn, 1972). Swimming patterns are poorly understood, it is known that *Entobdella*, *Diplozoon* and *Discocotyle* usually swim in a spiral path although *Discocotyle* may swim in a straight path, as do *Pseudodiplorchis* and *Protopolystoma*, the former swimming almost continuously, the latter making abrupt turns (Llewellyn, 1972; Tinsley & Earle, 1983; Tinsley & Owen, 1975). Parasites, such as *P.integerrimum*, may also possess hatching rhythms which are closely linked to host behaviour (MacDonald & Combes, 1978). Only a single confirmed case of host location by chemoreception has been documented, Kearn (1967) found that the oncomiracidia of *Entobdella soleae* recognised the mucus of its piscine host. Kearn (1980) noted that, in the absence of host mucus, the oncomiracidia of *E.soleae* show patterns of both positive and negative phototrophism, which in conjunction with water currents could produce search patterns. The combination of behaviours and receptors possessed by each particular parasite species must play a role in their location and distribution amongst the host population.

Anderson *et al.* (1978) applied a stochastic model to their data which demonstrated that a small variation in host susceptibility (including behavioral

traits) could generate patterns of dispersion similar to those observed experimentally. However, if susceptibility between hosts remained equal, then distributions would tend to be random. Therefore, in this study, as the tadpoles remained stationary for the majority of the infection period, behaviour may not have been a major factor, leading to the random/underdispersed patterns observed.

The use of the variance-mean ratio as an indication of the degree of overdispersion should be noted in the context of recent findings. The measure of overdispersion widely used in the literature, k, has been found not to be independent over a range of population densities (Taylor, Woiwod & Perry, 1979). Scott (1987) commented that the variance-mean ratio was '..a better measure of the degree of aggregation, whereas the parameter k provides information about the spread of the data around the mean'.

By controlling and/or moderating a number of factors it is apparent that the parasites in this study distributed themselves in a random to underdispersed manner. Llewellyn (1957) noted that the oncomiracidia of *P.integerrimum* made apparently random collisions with tadpoles, a pattern which is in agreement with the findings of Anderson *et al.* (1977). Anderson *et al.* (1978) suggested that, in nature, hosts will inevitably be exposed to aggregations of infective stages, the severity of infection being dependent on host behaviour. A clear example was described by Bychowsky (1957) who noted that tadpoles which drifted 2-3m from their hatching site (and the region of parasite egg deposition) remained uninfected by *P.integerrimum*, whereas those remaining close to the natal site became

infected. Thus, in a similar manner to that described for *Tylodelphys podicipina* by Kennedy (1981), as infection levels build, the initially random distribution may then tend towards overdispersion.

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# Oncos	Container	Volume /ml	Replicates
1	5cm petri	5	10
2	5cm petri	5	10
5	5cm petri	5	10
10	5cm petri	5	10
1	5cm petri	10	10
2	5cm petri	10	10
5	5cm petri	10	10
10	5cm petri	10	10
1	5cm petri	15	10
2	5cm petri	15	10
5	5cm petri	15	10
10	5cm petri	15	10
1	8.5cm petri	15	10
2	8.5cm petri	15	10
5	8.5cm petri	15	10
10	8.5cm petri	15	10
1	8.5cm petri	30	10
2	8.5cm petri	30	10
5	8.5cm petri	30	10
10	8.5cm petri	30	10
1	8.5cm petri	45	10
2	8.5cm petri	45	10
5	8.5cm petri	45	10
10	8.5cm petri	45	10

Table 9.1 Exposure protocol for single *Hyla versicolor* tadpoles (n = 240) to the oncomiracidia of *Polystoma nearcticum*.

A.

# Oncos	Container	Volume/ml	Replicates
2	8.5cm petri	30	10
4	8.5cm petri	30	10
10	8.5cm petri	30	10
20	8.5cm petri	30	5
2	8.5cm petri	45	5
4	8.5cm petri	45	10
10	9.5cm dish	45	5
20	9.5cm dish	45	5
2	9.5cm dish	70	5
4	9.5cm dish	70	5
10	9.5cm dish	70	5
20	9.5cm dish	70	5
2	9.5cm dish	115	5
4	9.5cm dish	115	5
10	9.5cm dish	115	5
20	9.5cm dish	115	5

B.

# Oncos	Container	Volume/ml	Replicates
5	9.5cm dish	70	5
10	9.5cm dish	70	5
5	7x14cm aquaria	80	5
10	7x14cm aquaria	80	5
5	7x14cm aquaria	120	5
10	7x14cm aquaria	120	6

Table 9.2 Exposure protocol of multiple exposures of **A.**) pairs of tadpoles (n = 200) and **B.**) groups of five (n = 155).

9.7 Legends.

Fig.9.1 Relationship between the prevalence of infection and oncomiracidial density for single tadpole exposures. Symbols: ● = 5cm diameter petri dish exposures, ∇ = 8.5cm diameter petri dish exposures.

Fig 9.2 Relationship between mean worm burden per tadpole and the initial oncomiracidial density for all replicates of single tadpole exposures (R^2 = 55.1%, F = 26.99, $p < 0.0001$).

Fig 9.3 Relationship between mean worm burden per tadpole and the initial oncomiracidial density for **A.**) all replicates of tadpoles exposed in pairs (R^2 = 63.0%, F = 23.80, $p < 0.0001$) & **B.**) all replicates of tadpoles exposed in groups of five (R^2 = 60.7%, F = 6.17, p = 0.068 NS).

Fig 9.4 Comparison of regression analyses for the 3 host densities in relation to the total number of parasites recovered and the initial oncomiracidial density.

Fig.9.5 Relationship between the nose to tail-tip length and body weight of 104 tadpoles (R^2 = 97.1%, F = 3446.55, $p < 0.0001$).

Fig.9.6 Relationship between the mean values of the host size - invasion success index and initial oncomiracidial density. Symbols: + = mean, ▮-▮ = standard deviation.

Fig.9.7 Relationship between the mean values of the distribution index for tadpoles exposed in pairs and initial oncomiracidial density. Symbols: + = mean, ▮-▮ = standard deviation.

Fig.9.8 Relationship between the variance/mean ratio (s^2/x) and initial oncomiracidial density for tadpoles exposed in groups of five.

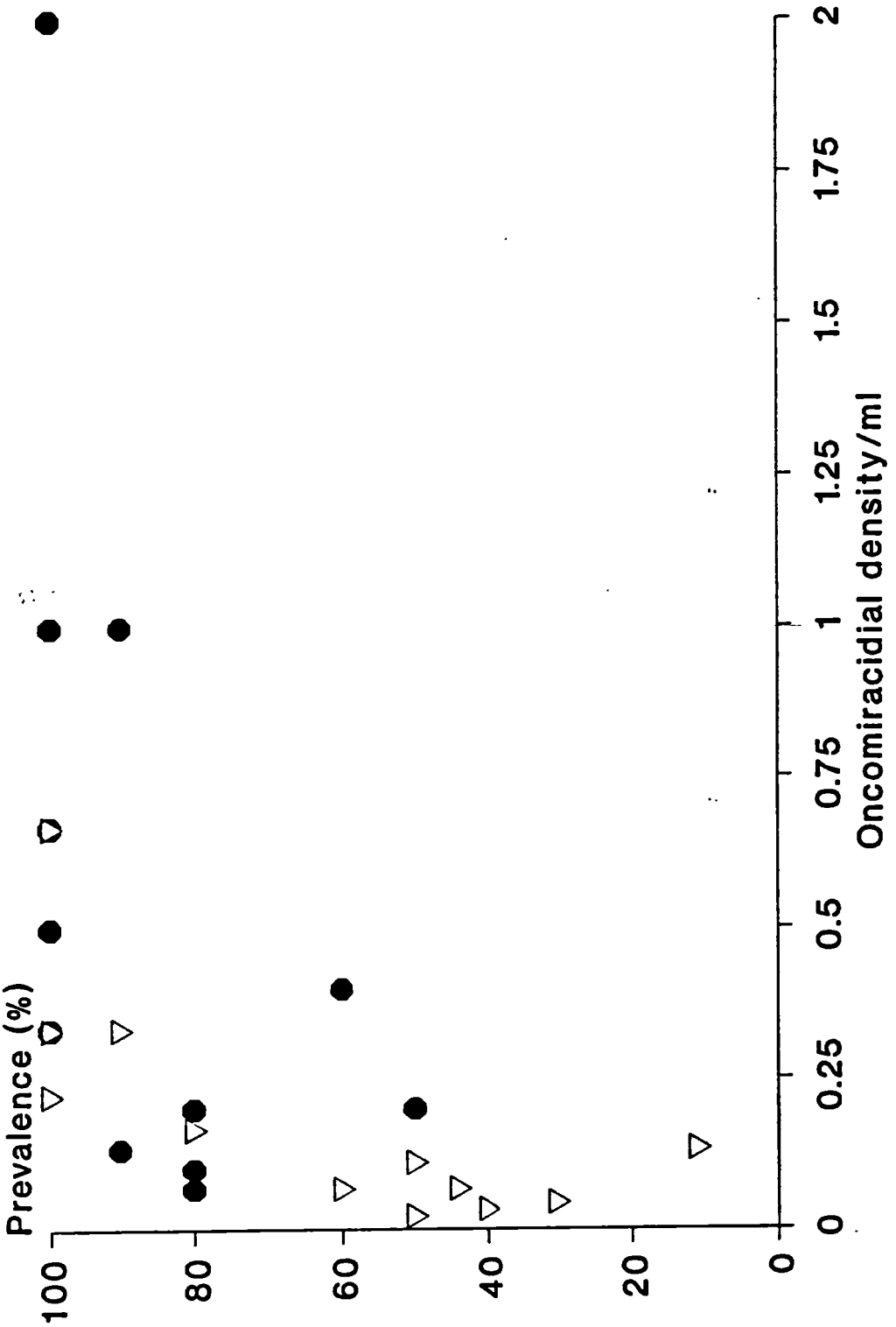
Prevalence (%)
100
80
60
40
20
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Oncomiracidial density/ml

Fig.9.1

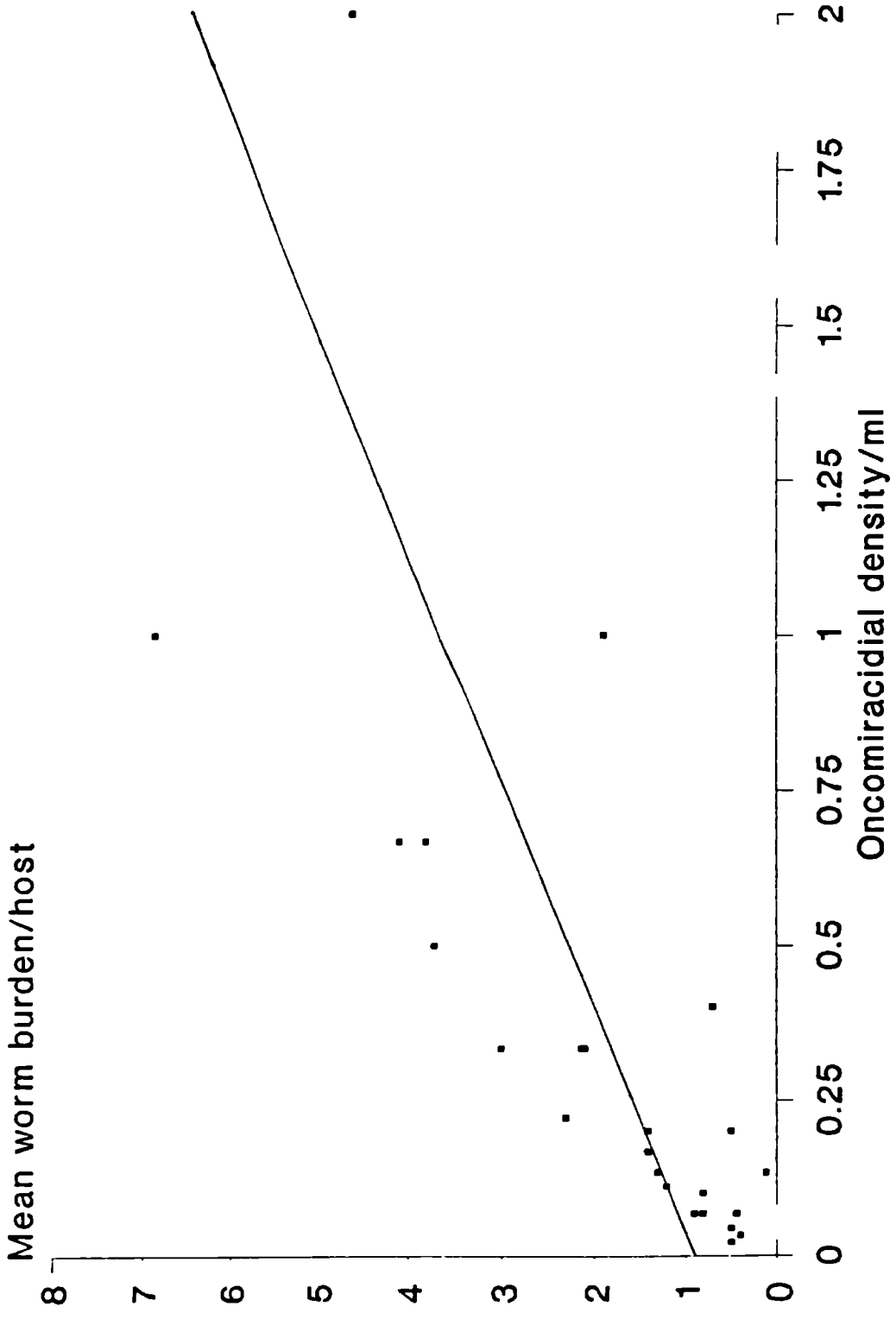
Mean worm burden/host
8
7
6
5
4
3
2
1
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Oncomiracidial density/ml

Fig 9.2

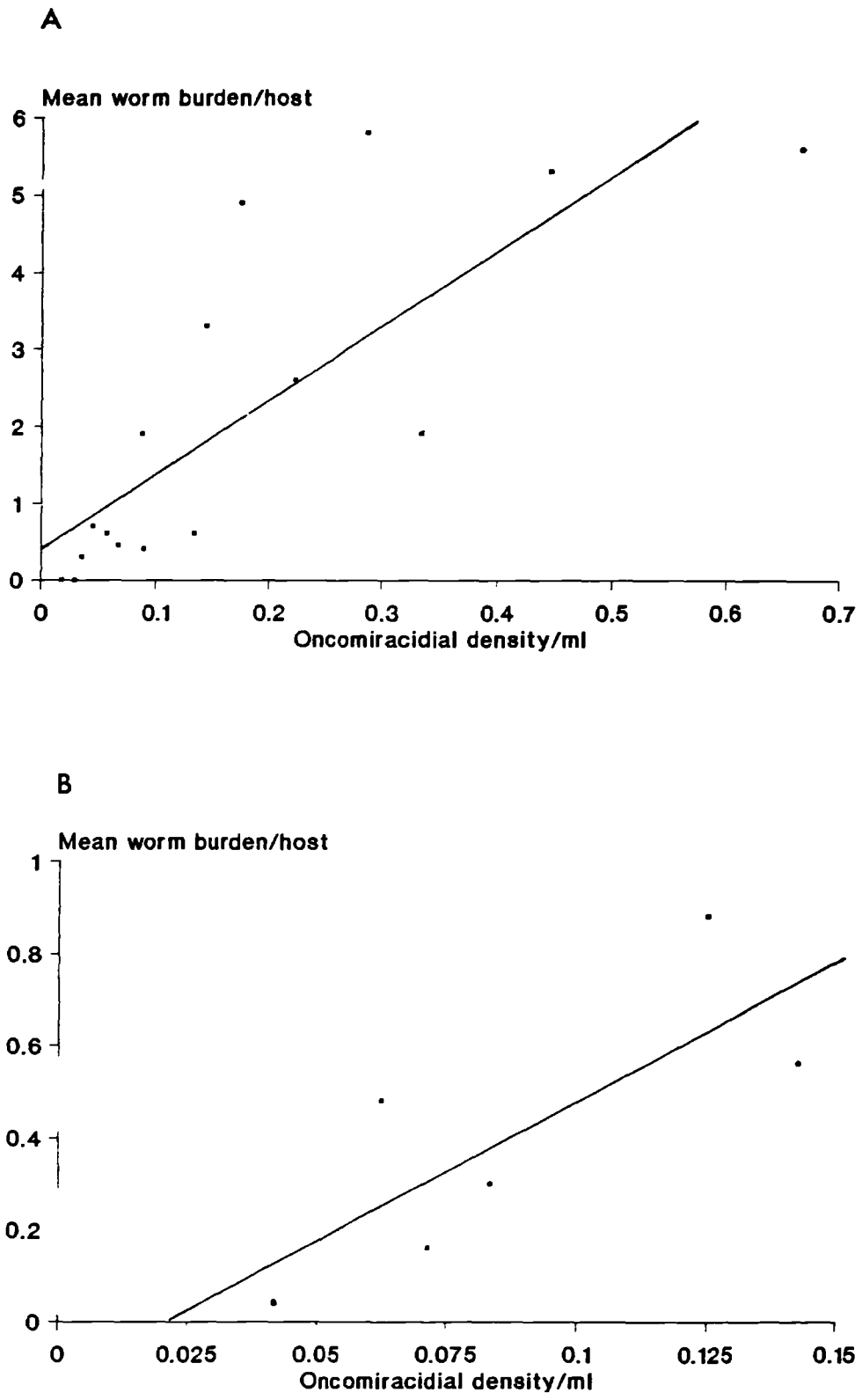
A
Mean worm burden/host
6
5
4
3
2
1
0
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Oncomiracidial density/ml
B
Mean worm burden/host
1
0.8
0.6
0.4
0.2
0
0
0.025
0.05
0.075
0.1
0.125
0.15
Oncomiracidial density/ml

Fig 9.3

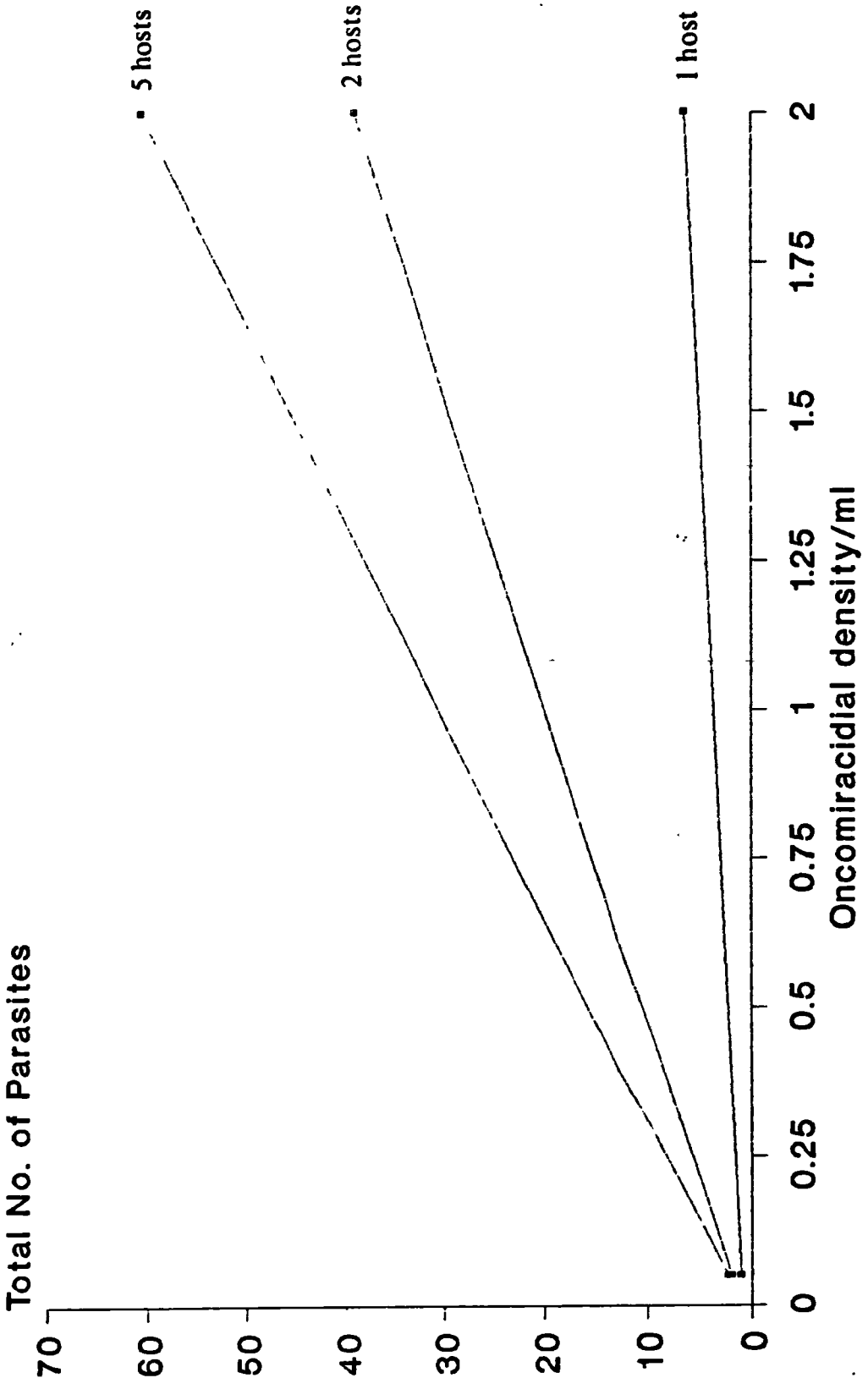
Total No. of Parasites
70
60
50
40
30
20
10
0
0
0.25
0.5
0.75
1
1.25
1.5
1.75
2
Oncomiracidial density/ml
5 hosts
2 hosts
1 host

Fig 9.4

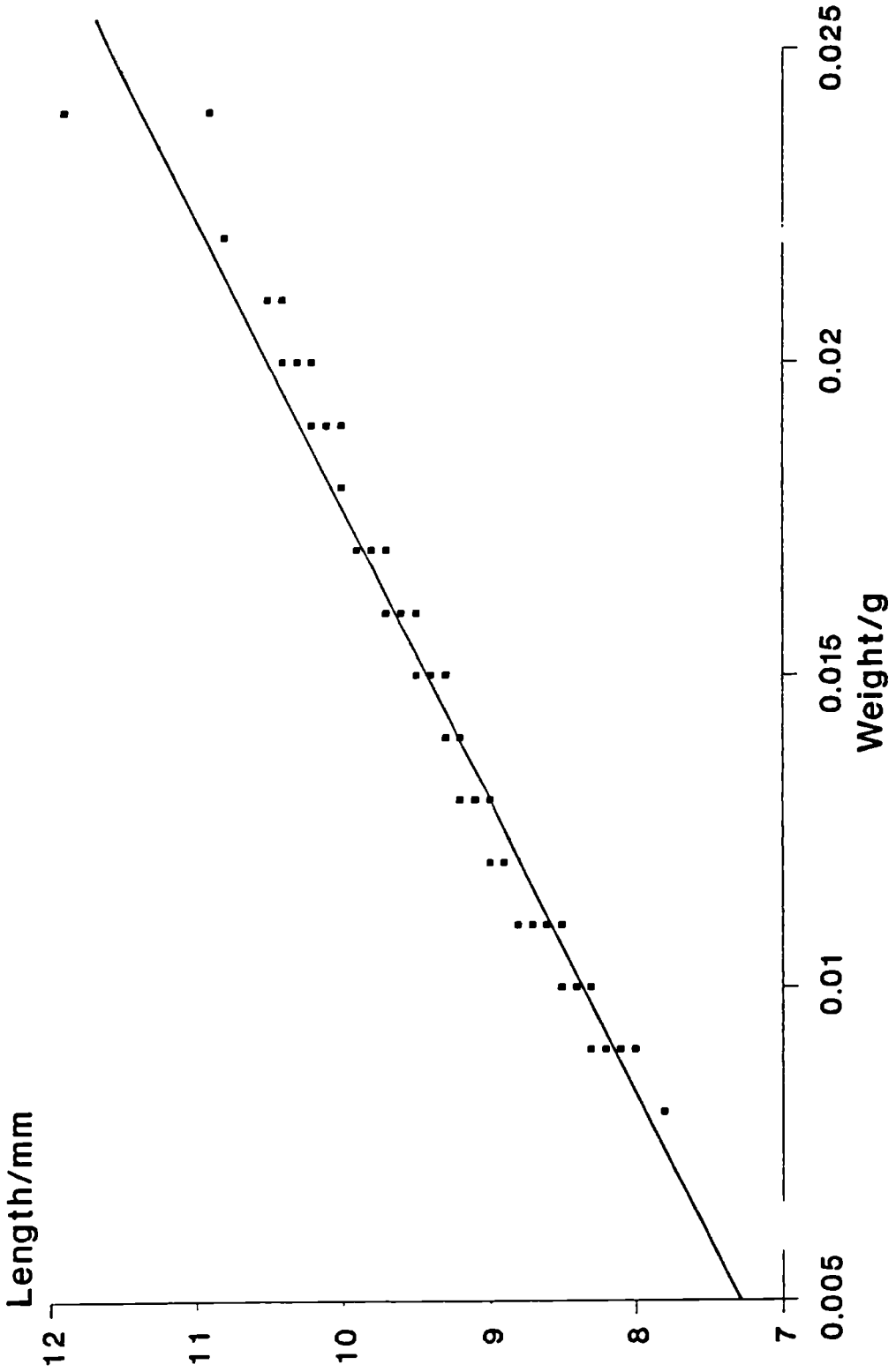

Fig.9.5

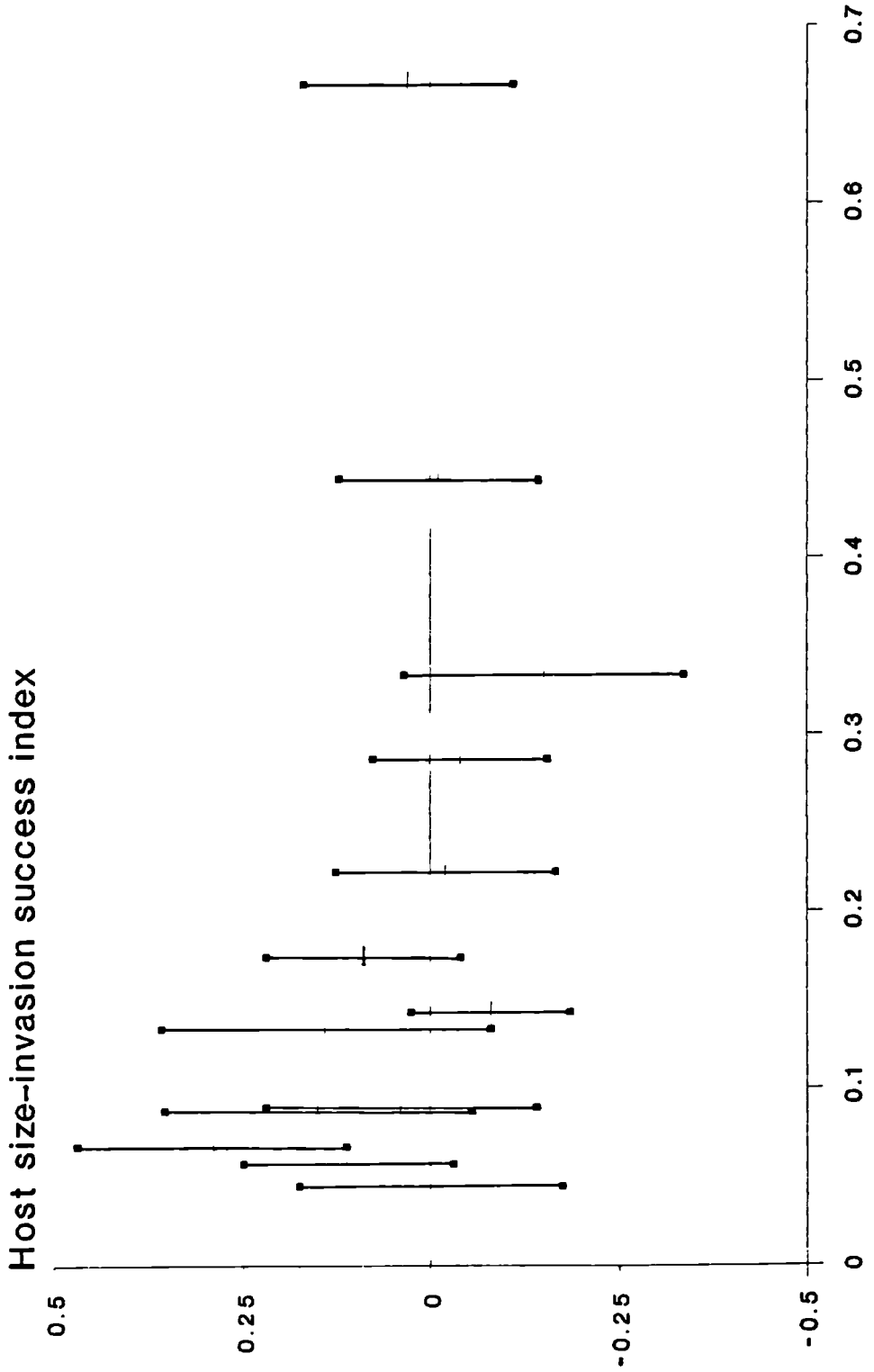
Host size-invasion success index
0.5
0.25
0
-0.25
-0.5
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
Oncomiracidial density/ml

Fig.9.6

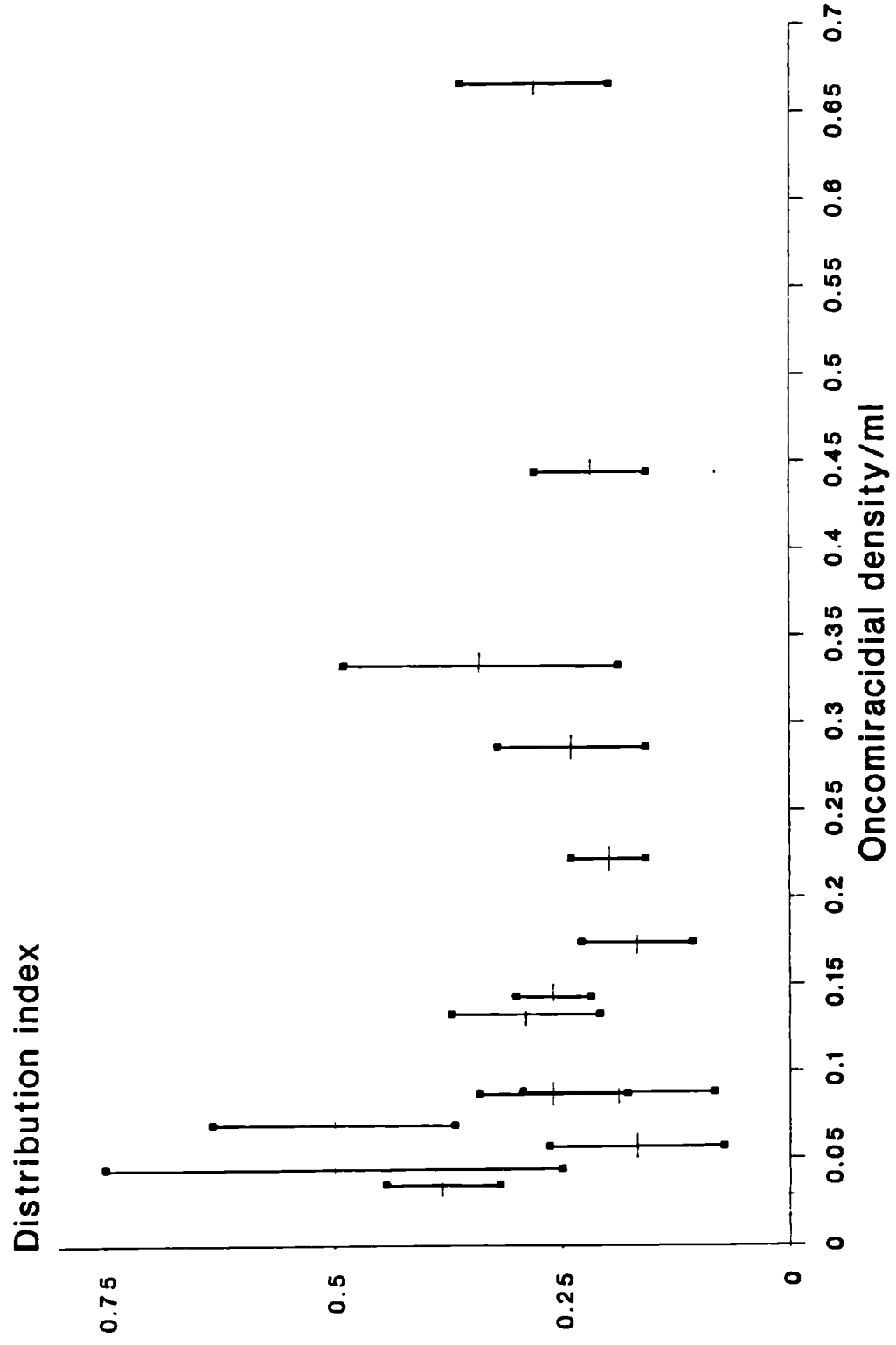
Distribution index
0.75
0.5
0.25
0
0
0.05
0.1
0.15
0.2
0.25
0.3
0.35
0.4
0.45
0.5
0.55
0.6
0.65
0.7
Oncomiracidial density/ml

Fig.9.7

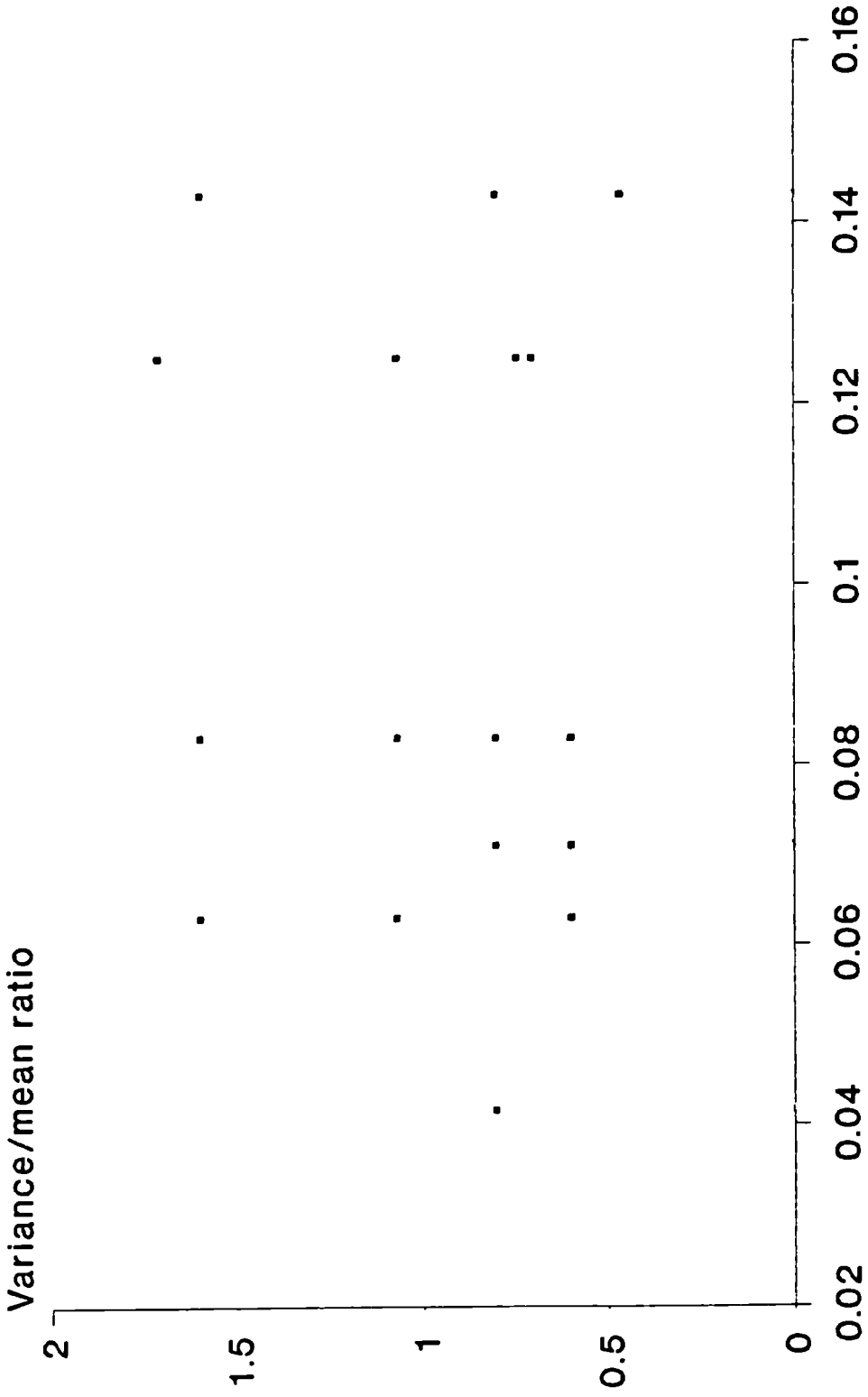
Variance/mean ratio
2
1.5
1
0.5
0
0.02
0.04
0.06
0.08
0.1
0.12
0.14
0.16
Oncomiracidial density/ml

Fig.9.8

Chapter 10.

A Helminthological Survey of the Fire and Yellow-bellied toads, *Bombina bombina*, *B.variegata* (Anura:Discoglossidae) and their hybrids from the former Yugoslavia.

10.1 Abstract.

The fire-bellied toad, *Bombina bombina* and the yellow-bellied toad *B.variegata*, form a narrow hybrid zone throughout eastern Europe, where elevations lie between the preferred habitats of pure populations. A total of 110 toads were collected at 6 sites from within the zone, near Zargreb in the former Yugoslavia. Specimens were electrophoretically typed and a genetic index produced for each individual. A full helminthological survey of 29 *B.bombina*, 45 *B.variegata* and 36 hybrids revealed that both pure and hybrid populations shared a common fauna. Four major parasitic groups were represented: *Diplodiscus subclavatus*, *Gorgoderina alobata*, *Haematoloechus sp.* and *Opisthoglyphe ranae* (Digenea); larval cysts (Cestoda); *Cosmocerca ornata*, *Heduris androphora*, *Oswaldocruzia filiformis*, *Rhabdias bufonis*, unidentified larval cysts (Nematoda) and *Acanthocephalus ranae* (Acanthocephala). This survey documents a number of new host and/or locality records.

No significant relationship between parasitic infection and a suite of host factors (which might indicate parasite-induced pathology) was found for either pure or hybrid hosts, except for the *C.ornata*. At one locality, regression analysis revealed a significant positive correlation between host body weight and infection by *C.ornata*; $R^2 = 21.9\%$, $p = 0.021$ for *B.bombina* and $R^2 = 38.2\%$, $p = 0.014$ for hybrids. Statistical analyses (Mann-Whitney tests) were undertaken for the 6 most common parasite species at the two largest collection sites. Only *C.ornata* and *G.alobata* had significantly different distributions between sites ($P < 0.05$). Furthermore, there was no evidence for differences in distribution for any parasite species between pure and hybrid genotypes. The helminth fauna is discussed in

relation to host genetics, ecology, and geographical range, in addition to parasitic transmission mechanisms. A checklist of the recorded helminth fauna of *B.bombina*, *B.variegata* and their hybrids has been compiled. In addition, a list of the helminth fauna of amphibians documented from the former Yugoslavia is included.

10.2 Introduction.

There are six species in the genus *Bombina*, which belongs to the anuran family Discoglossidae. The genus was first described by Oken in 1816 and now has representatives in Europe, Turkey, western USSR and the eastern Asia (Duellman & Trueb, 1986). Two members of the genus, the fire-bellied toad, *Bombina bombina* (Linnaeus, 1761) and the yellow-bellied toad, *B.variegata* (Linnaeus, 1758) are common in eastern Europe. The ranges of both species meet from Poland to the Black Sea (Fig.10.1). There is a zone of overlap throughout the contact of each species range, with a breakdown in reproductive isolation. These two species are the most closely related in the genus (Szymura, 1988) and hybrid zones have primarily been mapped in Poland (Szymura 1976a, 1976b, 1988; Szymura & Barton, 1986, 1991). However, the zone is also present in a number of other European countries, namely Austria, Bulgaria, Czechoslovakia, Hungary and Yugoslavia (Gollmann, 1984, 1986, 1987; Gollmann, Roth & Hodl, 1988).

Although closely related, there are marked differences in the ecology of the toads. *B.bombina* is a lowland species found in northern and central Europe and *B.variegata* is found in the upper elevations of western, central and southern Europe (Arntzen, 1978). Typically, *B.bombina* lives in the vicinity of large water

courses and breeding is prolonged. Inhabiting more montaine regions, *B.variegata* is relatively more terrestrial, tending to utilise small or temporary pools which are prone to drying (Medej, 1973; Barandun, 1991; Rafinska, 1991). Furthermore, Rafinska (1991) found significant differences in the reproductive strategies of *B.bombina* and *B.variegata*. The latter lays smaller clutches of larger and heavier eggs which, once hatched, develop at the same rate as *B.bombina* but metamorphose on average 10 days earlier. Rafinska (1991) regarded this as an adaptation to the more ephemeral nature of their primary choice of habitat. Information on the number of times a male may breed are scarce but Seidel (1987) stated that although *B.variegata* may breed 2 to 3 times per annum only 6% of females produce more than one clutch a year.

Both species not only differ in ecology, but also in DNA content (Olmo *et al.*, 1982), immunology (Maxson & Syzmura, 1984), mtDNA divergence (Syzmura, Spolsky & Uzzell, 1985) and electrophoretic profile (Szymura, 1976a). Szymura (1976a) first described the polymorphic isoenzyme patterns for *B.bombina* and *B.variegata* (lactate dehydrogenase - Ldh-1; malate dehydrogenase - Mdh-1; creatine kinase - Ck; adenylate kinase - Ak and glucosephosphate isomerase - Gpi). Szymura & Farana (1978) investigated the inheritance and linkage of these enzyme loci. From laboratory crosses it was discovered that for each enzyme system, offspring inherited codominant alleles at independent loci. Segregation ratios were consistent with normal Mendelian inheritance with no apparent linkage between genes.

Genetic analyses of the hybrid zone in Poland suggest that there is a dynamic interaction between two dominant processes (Szymura & Barton, 1986, 1991). Gene flow into the zone from adjacent pure populations opposes selection pressures against the hybrids at a large number of loci. Selection against hybrids is indicated by embryonic mortality and morphological aberrations. The zone is approximately 20km wide (Szymura, 1976b) with coincident enzyme clines changing over 6km followed by long tails of introgression. The enzyme clines are also coincident with morphological and mtDNA clines. As the transition between lowland and mountainous regions is much wider than 6km, clines are not thought to be linked to habitat (Szymura & Barton, 1986). Moreover, the zone appears to be ancient and without reference to human disturbance. Szymura & Barton (1986) suggested that the reproductive isolation mechanisms have evolved in many small steps. In certain areas of sympatry, such as Yugoslavia, mating preferences have allowed the two species to retain some degree of isolation with a bimodal distribution of the electrophoretic hybrid index (Szymura & Barton, 1986). After further analysis of the Polish zone, Szymura & Barton (1991) proposed that the apparent long range migration had little influence on the shape of the clines. The transition in habitat where the toads come into contact ranges from 110m above sea level at Zargreb, Yugoslavia (Syzmura, 1988) to 300-400m in Austria (Gollmann, 1984). Where data are available, it appears that the zone has not moved over the last 30-80 years (Szymura & Barton, 1991).

There is variation in the formation of the zone, with 3 distinct patterns; smooth clines, mosaic hybrid zones and remnants of ancient hybridisation. Smooth clines

such as those described above (Szymura & Barton, 1986, 1991) can be found in Poland and northwestern Yugoslavia. Mosaic zones were also described from Yugoslavia, with both parental types and an unusual abundance of F_1 hybrids found in sympatry in the centre of the transect (Syzmura, 1988). Relic populations found in Austria and Yugoslavia have introgressed alleles but are separated from the mosaic type by possessing genotype distributions close to Hardy-Weinberg predictions (Gollmann, 1984; Syzmura, 1988). Therefore, although mapping and examination of the hybrid zone is well documented, some of the dominant processes which must drive the introgression, i.e. the ecology and reproductive biology are not well understood (Madej, 1973; Rafinska, 1991).

A diverse helminth fauna has been recorded from *B.bombina*, *B.variegata* and their hybrids: with 36 species from Czechoslovakia (Vojtkova, 1982); 24 species from the former USSR (Ryzhikov, Sharpilo & Shevchenko, 1980); 11 species from Albania (Prokopic, 1960); 10 species from Bulgaria (Batchvarov, 1965, 1972; Boschkov, 1965); 6 species from Yugoslavia (Rozman, 1976); 5 species from Denmark (Frandsen, 1974) and 15 species from Poland (Grabda-Kazubska & Lewin, 1989). Furthermore, Lees & Mitchell (1966) described a new species of *Gorgoderina* from *B.variegata* in Austria. The helminth fauna of *B.bombina* and *B.variegata* has been complied into Table 10.1, with the designation of authorities and species nomenclature as described by the original authors.

According to a review of European parasitological research of the amphibia (Vojtkova, 1990), *B.variegata* has received less attention than its lowland

counterpart. A total of 1201 *B.bombina* have been examined for helminth infection as opposed to 456 *B.variegata*. However, Vojtkova (1990) did not include the work of Rozman (1976) which incorporated a further 51 *B.bombina* and 129 *B.variegata* collected from various regions of Yugoslavia.

Regarding Yugoslavia, Vojtkova (1990) cited the work of Questel (1942), who surveyed 51 *B.bombina* and recovered 6 species of parasite. Other amphibians have been examined by Hristovski & Lees (1968), Hristovski (1969 & 1970), Hristovski & Riggio (1971) and Hristovski & Lees (1973). Furthermore, Rozman (1976) dissected 1,283 anurans including the 51 *B.bombina* and 129 *B.variegata*. At present, nine species of Amphibia have been investigated in Yugoslavia with 30 helminth species recovered (Rozman, 1976; Vojtkova, 1990). These are summarised in Table 10.2.

Hybrid zones provide interesting implications for susceptibility to parasite infection as field studies of wild populations of mice in Europe have documented significant parasite burdens in hybrids in comparison to pure bred hosts sampled from outside the zone (Sage *et al.*, 1986; Moulia *et al.*, 1991). A similar phenomenon has also been recorded for fish (Dupont & Crivelli, 1988). Alternatively, the work of Coustau *et al.* (1991) and LeBrun *et al.* (1992) indicates that a particular parental host genome may be able to resist infection by specific parasites; therefore, the selective pressure may favour the non-susceptible genome (and their closely associated hybrids), displacing the competing (susceptible) species. By experimental studies, genetic predisposition to infection by intestinal

nematodes and cestodes has been demonstrated for hybrid mice (Moulia *et al.*, 1993; Munger *et al.*, 1989; Wassom *et al.*, 1973, 1974, 1986). Predisposition of this nature will have a significant influence on the survival and competitive ability of hybrid genotypes and may restrict the outward diffusion of hybrid characters into the surrounding populations of pure species. Therefore, if susceptibility to helminth infection exists in the *B.bombina/B.variegata* hybrid zone, then this may be a contributory factor in selection against hybrids. The parasites recovered from this study may not only provide a further evaluation of the species present in European *B.bombina* and *B.variegata* but also a comparison between infection levels in pure and hybrid toads in sympatry.

10.3 Materials and Methods.

A total of 110 toads were collected at 6 breeding ponds within the hybrid zone, close to Zargreb, in the former Yugoslavia by Dr. N. Sanderson (University of East Anglia). The genotypic frequencies at each site are listed below with sample size in brackets.

1). Hybrid genotypes (n = 9).

2). *B.bombina* (n = 3), *B.variegata* (n = 44) and hybrid genotypes (n = 5).

3). *B.bombina* (n = 2) & hybrid genotypes (n = 3).

4). Hybrid genotypes (n = 3).

5). *B.bombina* (n = 24) & hybrid genotypes (n = 15).

6). *B.variegata* (n = 1) and hybrid genotypes (n = 3).

Prior to fixing, the toads were toe-clipped for electrophoretic typing. The cut appendage was then stored at -20^{0}C until return to -70^{0}C facilities in the laboratory at Norwich. In the field, the toads were anaesthetised, pithed and fixed in a 10% formal saline solution by Dr. Sanderson. The specimens were washed and transferred to 70% alcohol by the author after their return to the U.K.

The specimens were identified by an individual reference number attached at the time of fixation. Toads were dissected by the author as described in Chapter 3. All major organ systems were examined with a dissection microscope for possible parasitic infection, (particularly the mouth, nostril sinuses, male vocal sac, eustachian tubes, lungs, alimentary tract, urinary bladder and kidneys). Furthermore, to locate encysted parasites, all soft tissue was teased apart (including the epidermis and muscle blocks). No blood samples were taken. All helminths were removed and their location recorded. For encysted nematodes and cestodes, only their presence was recorded. Digeneans and acanthocephalans were stained, dehydrated and mounted *in toto* as described in Chapter 3. However, as the parasites were fixed *in situ* without coverslip pressure, they were flattened prior to processing (typically using a glass slide). Nematodes were temporarily mounted in glycerol for identification. All parasites were identified to genus and where possible to species.

Electrophoresis was undertaken at the University of East Anglia by Dr N.Sanderson using the method described by Syzmura (1976a) and Syzmura & Farana (1978). The toes provided sufficient material to score 5 loci and a genetic

index was produced. Those animals scoring a *B.bombina* locus = 0, hybrids = 1 and *B.variegata* = 2, therefore pure *B.bombina* scored 0 points for 5 loci and *B.variegata* scored 10 points, hybrids scoring in between these extremes.

10.4 Results.

The range of parasite species recovered from the 110 specimens of *B.bombina*, *B.variegata* and their hybrids is recorded in Table 10.3. The parasite fauna at the 6 collection sites is summarised by host genotype in Table 10.4. F_1 and backcross genotypes were recovered, but because of the small sample sizes collected, no distinction has been made in this study. Non-parametric statistical tests (Table 10.7) and frequency distributions were based on the 3 largest collections, i.e. *B.variegata* at site 2 (n = 44), *B.bombina* (n = 24) and hybrid genotypes (n = 15) at site 5. There are a number of new species and/or locality records for parasites infecting *Bombina spp.* in Yugoslavia.

The digenean *Diplodiscus subclavatus* (Goeze, 1782) Diesing, 1836 was recovered from the rectum of pure and hybrid genotypes at two sites. The prevalence of infection for *B.variegata* was 4.5% at site 2, 26.7% for hybrid genotypes at site 5 and ranged from 12.5 to 66.6% between sites 2 & 5 for *B.bombina* (Table 10.4). Worm burdens also exhibited wide variation between sites. At pond 2, the two *B.variegata* infected with *D.subclavatus* harboured 80 & 83 parasites. *B.bombina* at site 5 had burdens ranging from 1 to 8 parasites/host and hybrid genotypes 1-118; however, only 1/7 of infected specimens at this site harboured more than 8 *D.subclavatus*. Therefore, the frequency distribution of

infection levels for *D.subclavatus* is positively skewed. Individual Mann-Whitney tests between the 3 largest pure and hybrid collections revealed no significant differences in the distribution of *D.subclavatus* either between sites or hosts genotypes (Table 10.7B). This digenean has not previously been recovered from *B.bombina* or hybrid genotypes in Yugoslavia.

Gorgoderina alobata Lees & Mitchell, 1966 was recovered from the urinary bladder of pure and hybrid host genotypes. Infected hosts were found at 4/6 sites, with prevalences ranging from 12.5 to 52.3%. Heavier worm burdens were found at site 2, in both pure *B.variegata* and hybrid genotypes. Frequency distribution plots (Fig.10.2A) display the higher intensity of infection at site 2. For pure-bred hosts frequency distributions were positively skewed and approached a poisson distribution for hybrids. Individual Mann-Whitney tests indicated significant differences in the distribution of *G.alobata* between hosts at sites 2 and 5, but not between hosts at site 5 (Table 10.7C). This finding presents a new species record for pure and hybrid *Bombina spp.* in this locality.

Lung flukes of the genus *Haematoloechus* Looss, 1899 were found at 3/6 sites infecting both pure and hybrid toads. Prevalences were 20.5% for *B.variegata* at site 2, 26.7% for hybrid genotypes at site 5, 50.0% and 16.7% for *B.bombina* at sites 3 and 5 respectively. The maximum worm burden was 11 parasites/host, however; in the 3 largest collections the mean intensity of infection ranged from 1.3 to 3.0 parasites/host (Table 10.4). Frequency distribution plots (Fig.10.2B) are positively skewed for pure-bred hosts and close to a poisson distribution for the

hybrid sample. Individual Mann-Whitney tests revealed no significant differences in the distribution of *Haematoloechus sp.* either between sites or hosts genotypes (Table 10.7D). Identification to species was not possible as the robust nature of these worms did not allow suitable preparations to be made. This represents the first record of members of the genus *Haematoloechus* infecting pure and hybrid *Bombina spp.* in Yugoslavia.

Opisthoglyphe ranae (Frolich, 1791) Looss, 1899 was the least common digenean, only being present in *B.variegata* and hybrid genotypes at sites 1 & 2. Prevalences ranged from 6.8 to 22.2% with a maximum of 9 worms recovered from the intestine of a single host. Infection by this parasite of *B.bombina* x *B.variegata* hybrids has not previously been reported from Yugoslavia.

The acanthocephalan, *Acanthocephalus ranae* (Schrank, 1788) Luhe, 1911 was recovered from *B.bombina* and hybrid genotypes at only a single location, representing new host records at this locality. Prevalence of infection was 33.3% (3.1 worms/host) and 13.3 (3.5 worms/host) respectively. An individual Mann-Whitney test between pure *B.bombina* and hybrid specimens collected at site 5 indicated no significant difference in distribution (Table 10.7F).

Small numbers of unidentified larval cestodes were found encysted in pure *B.bombina* and *B.variegata* at sites 2 and 5. The cysts were found in the connective tissue surrounding the junction of the stomach and intestine.

Nematodes identified as *Cosmocerca ornata* (Dujardin, 1845) Raillet & Henry, 1916 were recovered from the lower alimentary tract of all host types sampled at 5/6 localities. Prevalences ranged from 33.3 to 50.0% (1-14 worms/host) for *B.bombina*, 86.4-100% (1-8 worms/host) for *B.variegata* and 46.7-100% (1-11 worms/host) for hybrid genotypes (Table 10.4). Frequency distribution plots (Fig.10.3A) indicate a broad spread of infection for pure-bred hosts, although distributions are positively skewed for all genotypes. Individual Mann-Whitney tests indicated significant differences in the distribution of *C.ornata* between *B.variegata* (sites 2) and hybrids (site 5), but not between pure hosts or *B.bombina*/hybrids (Table 10.7A). This is the first record of this nematode from hybrid genotypes in Yugoslavia.

Heduris androphora Nitzsch, 1821 were found firmly attached to the gastric mucosa of pure *B.bombina*, *B.variegata* and hybrid genotypes. This was a common nematode, recovered at all locations, with only *B.variegata* at site 2 uninfected. Prevalences ranged from 33.3 to 50.0% (1-25 worms/host) for *B.bombina*, 100% (3 worms/host) for *B.variegata* and 22.2-100% (1-16 worms/host) for hybrid genotypes (Table 10.4). Frequency distribution plots (Fig.10.3B) indicate a positively skewed distribution of *H.androphora* within *B.bombina* and hybrid genotypes at site 5. Furthermore, an individual Mann-Whitney test detected no statistical difference in infection at this location (Table 10.7E). This is the first record of *H.androphora* infecting this group of hosts in Yugoslavia.

The gut-dwelling nematode, *Oswaldocruzia filiformis* Goeze, 1782 was recovered in small numbers from *B.variegata* and hybrid individuals at 5/6 collection sites. Prevalences were 4.5% (2-11 worms/host) and ranged from 6.7 to 33.3% (1-10 worms/host) respectively. Both are new host records in this region.

The pulmonary nematode *Rhabdias bufonis* Schrank, 1788 was only recovered from 2 hosts at site 5, one pure *B.bombina* and one hybrid individual, both harbouring a single worm. Unidentified nematode larval cysts were recovered from 18.2% of *B.variegata* at site 2 and a single hybrid at site 1. The cysts were found embedded between the mucosa and outer musculature of the stomach, in addition to the external tissues of the intestine and rectum.

As all the toads were fixed under the same conditions, body and organ weights can be compared within the sample. For statistical analysis, the weight of the stomach contents at dissection were subtracted from the total body weight of each host. In addition to total body weight, other host factors were considered: snout-vent length (SVL), fat body and gonad weight, all of which may be affected by parasite-induced pathology. No significant correlations were found between this suite of host factors and parasitic infection, except for the nematode, *C.ornata* at site 5. For both pure *B.bombina* and hybrid specimens a significant relationship was recorded between host body weight and infection by this nematode (Fig.10.4A, R^2 = 21.9%, F = 6.18, p = 0.021 for *B.bombina* & Fig.10.4B, R^2 = 38.2%, F = 8.03, p = 0.014 for hybrids). A similar correlation was found for infection and host SVL (R^2 = 30.0%, F = 5.58, p = 0.034 for hybrids);

however, for pure genotypes this was not significant (R^2 = 14.3%, F = 3.68, p = 0.068 NS for *B.bombina*). The gradients of the regression lines indicate that there may be differences between pure and hybrid hosts with regards to the possible effect of *C.ornata* infection on body size. Further investigation would be required before any firm conclusions can be drawn.

Comparisons on the basis of host sex were made at the largest sampling sites (sites 2 & 5). No dimorphic variation was found between male and female toads (Table 10.5). Of the *B.variegata* collected at site 2, 20.5% (9/44) were female. At site 5, 12.5% (3/24) of *B.bombina* and 26.7% (4/15) of hybrids were female. The infection levels of the 8 most common helminths infecting each sex are summarised in Table 10.6. Although infection levels varied widely, sample sizes of female toads were too small for statistical comparison. Typically, the large samples (males), harboured a wider diversity of parasites.

10.5 Discussion.

The fire and yellow-bellied toads have been the subject of intensive helminthological investigation, primarily from authors in eastern Europe (see Table 10.1). However, this work has not been coordinated with the genetic studies of the hybrid zone and as a result only a limited number of confirmed hybrids have been examined, with few parasite species recorded. This study presents the first comparison between pure *B.bombina*, *B.variegata* and their hybrids.

The digenean *Diplodiscus subclavatus* (Goeze, 1782) Diesing, 1836 has often been assigned to Pallas 1760, however, Prudhoe & Bray (1982) and Sey (1990) considered *Planaria subclavatus* Goeze, 1782 to be the correct original description. Subsequently, Diesing (1836) placed this species in the subfamily Diplodiscinae Cohn, 1904. *D.subclavatus* has been reported from *B.bombina* in Czechoslovakia, Denmark, Germany, Poland, Rumania and the former U.S.S.R. and from *B.variegata* in Czechoslovakia, Switzerland and Europe (see Prudhoe & Bray, 1982). Rozman (1976) also recorded *D.subclavatus* from *B.variegata* in Yugoslavia. Sey (1990), in a major review of amphistomes, documented a broad host range for this parasite, with its distribution extending throughout Eurasia, from Poland to China. This distinctive amphistome was identified from accounts by Prudhoe & Bray (1982) and Sey (1990).

The life-cycle of *D.subclavatus* was first described in 1892 by Looss and Lang in separate and conflicting papers. Grabda-Kazubska (1980) produced a full description of the larval stages and confirmed the transmission mechanisms employed by *D.subclavatus*. Cercariae released from molluscan intermediate hosts typically encyst on the epidermis of the adult definitive host. It is the regular ingestion by the host of sloughed skin (infested with metacercariae) that is the principal infection route (cercariae have no affinity for tadpole skin). Another route, considered to be secondary by Grabda-Kazubska (1980), is by freely encysted metacercariae which are ingested by tadpoles. Metacercariae remain viable for only a few days and therefore may not accumulate in large numbers. The ingestion of skin bearing metacercariae is also the main transmission route

used by the amphistome *Megalodiscus temperatus* (Stafford, 1905) (see Chapter 8).

The flukes recovered from the urinary bladder were identified as members of the genus *Gorgoderina* as their testes were entire, rather the follicular forms characteristic of the *Gorgodera*. The body length, oral sucker to ventral sucker ratios, vitellaria and eggs correspond with the type description of *Gorgoderina alobata* rather than *G.vitelliloba*. *G.alobata* was first described by Lees & Mitchell (1966) from *B.variegata* in Austria. Subsequently, it has been suggested that this species is specific to the fire and yellow-bellied toads (Grabda-Kazubska, 1989). However, *G.alobata* has also been recorded from *Rana dalmatina* and *R.esculenta* in Czechoslovakia by Vojtkova (1972, 1974). *G.alobata* has been recovered from *B.bombina* in Czechoslovakia, Denmark and Poland, from *B.variegata* Austria, Czechoslovakia, Poland, Greece, Mecedonia, Slovenia and Yugoslavia and their hybrids in Czechoslovakia and Poland (see Prudhoe & Bray, 1982; Grabda-Kazubska & Lewin, 1989).

No life-cycle has been elucidated for *G.alobata*, however, the closely related *G.vitelliloba* has been examined in detail. Adult flukes produce embryonated eggs which pass out with host urine. Miracidia emerge in daylight and penetrate lamellibranch molluscs. Following asexual reproduction, cercariae are released, which either penetrate tadpoles or aquatic insect larvae and encyst. Transmission to the definitive host involves the consumption of infected tadpoles/insects. Excystment occurs only in the gut of adult anurans, with juvenile flukes migrating

to the kidneys via the ureters. After 21 days, migration to the urinary bladder begins, all reaching this site by 28 days. Sexual maturity ensues with eggs produced after 42 days p.i. with individual infection lasting up to 8 months (Smyth & Smyth, 1980). In the kidney, juvenile flukes digest cells extracellularly using their penetration glands. Adults in the urinary bladder feed on loose cells and small quantities of blood. Pathological damage to the kidneys may be sufficient to kill tadpoles and recently metamorphosed adults (Smyth & Smyth, 1980).

Gassmann (1972) indicated discriminate characters between *Opisthoglyphe ranae* (Frolich, 1791) and *Opisthoglyphe rastellus* (Olsson, 1876). On the basis of these characters (regarding testis, vitellaria, eggs and pharynx), the intestinal digenean recovered in this survey was identified as *O.ranae*. Grabda-Kazubska (1967) confirmed that *O.ranae* incorporated *O.endoloba* (Dujardin, 1845). *O.ranae* has been reported to infect *B.bombina* in Czechoslovakia, Poland, Rumania and the U.S.S.R., as well as *B.variegata* in Albania, Czechoslovakia, Macedonia (see Prudhoe & Bray, 1982) and Yugoslavia (Rozman, 1976).

O.ranae is a common intestinal parasite of European anurans. The life-cycle may take two courses, a 'normal' or an 'abbreviated' path (Joyeux & Baer, 1953). Eggs released with host faeces hatch in water and the miracidia penetrate molluscan intermediate hosts (lymnaeid or planorbid snails). Development continues in the digestive glands, the cercariae released may infect snails, amphipods, tricopterans and ephemerids, as well as adult frogs and tadpoles. Grabda-Kazubska (1969) found that cercariae invade tadpoles at any point on their

body, whereas with adult anurans they typically move over the body surface to the mouth, encysting around the oesophagus. From this position they become dislodged and continue development in the digestive tract. Therefore, the 'normal' life-cycle involves the invasion of tadpoles which fall prey to adult anurans (perhaps after their metamorphosis), the 'abbreviated' life-cycle involves the direct infection and encystment in adults. Unusually, this species only requires 24 hours as a metacercariae prior to successful establishment as an adult. Grabda-Kazubska (1969) concluded that the 'abbreviated' cycle is simply due to a single amphibian performing the role of both second intermediate and definitive host.

Lung flukes of the genus *Haematoloechus* are common in the fire and yellow-bellied toads, with 10 species and sub-species (although some are considered synonyms) recorded in Table 10.1A. Unfortunately in this study, identification to species was not possible because of difficulties in establishing the taxonomic characters of worms fixed *in situ* inside the host. *H.abbreviatus* (Bychowsky, 1932) is proposed by Grabda-Kazubska (1989) and Prokopic & Krivanec (1974) to be species specific for *Bombina spp.* however, Rozman (1976) recorded only *H.variegatus* from his Yugoslavian survey. Members of the Haematoloechinae utilise molluscs and aquatic insects as intermediate hosts, final infection of the definitive host occurs after the insect is consumed as a prey item (Prudhoe & Bray, 1982).

The acanthocephalan *Acanthocephalus ranae* (Schrank, 1788) has been synonymised with *A.falcatus* (Frolich, 1789) by a number of authors (Dujardin,

1845, Luhe, 1911: cited by Grabda-Kazubska, 1962). However, Grabda-Kazubska (1962) confirmed the validity of the latter by the differing body form, hook morphology and egg dimensions. Furthermore, *A.falcatus* was found to have an exclusively montaine distribution. Gassmann (1972) proposed overall length, number of longitudinal rows of hooks, size of individual hooks and their morphology, development of vaginal sphincters, testicular shape/position and cement gland morphology as discriminative characters between the two species. On the basis of both these descriptions the acanthocephalan found in *B.bombina* and hybrid genotypes in this study was identified as *A.ranae*. *A.ranae* has been recorded from pure *B.bombina*, *B.variegata* and hybrid genotypes in Poland (Grabda-Kazubska & Lewin, 1989) and from *B.bombina* in Hungary (Matskasi *et al.*, 1990). Rozman (1976) recovered *A.ranae* in Yugoslavia but not from *Bombina spp.*. Eggs are released into the gut of the definitive host and into the external environment with host faeces. The mature acanthor is liberated from the egg once ingested by an intermediate host (either the isopod *Asellus aquaticus* or the amphipod *Gammarus pulex*). The acanthor bores into the haemocoel, developing into the resistant resting stage, the cystocanth (Smyth & Smyth, 1980). The life-cycle is completed when the amphibian host consumes infected crustaceans.

Nematodes of the genus *Aplectana* and *Cosmocerca* have been recorded from the fire and yellow-bellied toads. These gut-dwelling worms are similar morphologically, but can be distinguished on the basis of a characteristic of male *Cosmocerca*, the plectane. Rosette papillae borne on the plectanes are diagnostic of *Cosmocerca*. Grabda-Kazubska (1986) produced a description of female *C.ornata*

(Dujardin, 1845) to distinguish this species from the morphologically similar *Neyraplectana schneideri* (Travassos, 1931). It was found that the host of origin may have an influence on the overall dimensions of these helminths. Grabda-Kazubska & Tenora (1991) also published an SEM study of the closely related *C.ornata* and *C.commutata* (Diesing 1851). On the basis of these studies and type material, the worms recovered in the present study were identified as *Cosmocerca ornata* (Dujardin, 1845). The life-cycle of *C.ornata* has not been elucidated, however, females are viviparous. It is possible that direct reinfection via a percutaneous route occurs, although cosmocercids typically have a free-living phase. Baker (1989) commented on the number of different transmission strategies employed by the family Cosmocercidae, stating that oral infection was a common route '...given the food habits and biology of the hosts of the Cosmocercidae'.

Trichostrongyloids, characterised by possessing an ovijector and a well-developed copulatory bursa, formed the second group of intestinal nematodes. They are typically long and thin worms, with longitudinal cuticular ridges and are common parasites of anurans. *O.goegei* has been reported from anurans in Yugoslavia (Rozman, 1976) and *O.bialata* from pure *Bombina spp.* in Poland (Grabda-Kazubska & Lewin, 1989). Due to the morphological similarity, Moravec & Vojtkova (1974) proposed that *O.filiformis* encompassed the synonyms *O.bialata* and *O.goegei*. Grabda-Kazubska & Lewin (1989) gave no indication of their reasons for the maintenance of *O.bialata* as a separate species. Baker (1989) stated that members of the genus *Oswaldocruzia* often have primitive life-cycles. Eggs are unusually large containing large amounts of reserve material.

Development occurs in the external environment, with the 3rd larval stage ensheathed within the cuticle of the 2nd stage. Infection by the 3rd larva stage is thought to be percutaneous with somatic migration via the circulatory system and lungs.

A distinctive group of nematodes were found solely in the stomach. The females possessed a chitinous hook, which was deeply embedded into the gastric mucosa. The worms recovered in this study were identified to species level on the basis of the redescription of *Heduris androphora* (Nitzsch 1821) by Petter (1971) from the stomach of *Triturus vulgaris*. Female *H.androphora* are oviparous, producing eggs which, by the time of deposition, contain developing embryos. Eggs released into the environment are ingested by the isopod, *Asellus aquaticus*. 1st stage larvae are liberated in the digestive tract and penetrate into the haemocoel. Development through all larval stages occurs in the intermediate host and unusually, continues to include the young adult (prior to ingestion by the definitive host). Therefore, the amphibian is only necessary for the final stage of the life-cycle, the fertilization and maturation of eggs.

Pulmonary nematodes of the genus *Rhabdias* were recovered from only two toads in this study, one *B.bombina* and one hybrid at site 5. The worms were identified as *R.bufonis* (Shrank, 1788), which are a common amphibian parasite in Europe and is the only species reported to infect *B.bombina* and *B.variegata*. As described for *R.ranae* in Chapter 8, the life-cycle involves protandrous hermaphrodites inhabiting anuran lungs. Eggs are released from the lungs and pass

through the alimentary tract. After hatching in the duodenum, the 1st stage rhabditiform larvae accumulate at the cloaca, entering the external environment with host faeces. The life-cycle may take a number of different paths, homogonic development occurs after the larvae moult to 3rd stage filariform larvae, directly penetrating the definitive host. Alternatively, the hetergonic development path involves further free-living moults to produce sexual adults, which mate, and the female produces viviparous larvae which use their mother for a food source after her death. These larvae may then penetrate an anuran host. Baker (1989) suggested that the method of migration to the definitive site is similar to that of *R.ranae*. Baker (1989) cited the work Fuelleborn (1928) which documented that infective *R.bufonis* may use snails as paratenic hosts. Baker (1989) supported this, commenting that molluscs are common prey items for amphibians.

As discussed in Chapters 4 and 8, Brandt (1936) and Campbell (1968) proposed that the diversity of parasitic fauna was directly related to the ecology of their amphibian hosts. The more restricted fauna recorded for *B.variegata*, both in the literature and in this study, may indicate the smaller number of specimens examined but most probably, this is due to their semi-terrestrial ecology. Therefore, *B.variegata* will have a reduced exposure to aquatic-borne infective stages, a factor also implicated by Grabda-Kazubska & Lewin (1989). The waterbodies in which breeding takes place are typically ephemeral and may not contain suitable molluscan intermediate stages. From literature records (Table 10.1) both *B.bombina* and *B.variegata* are infected by 24 species of adult Digenea. However, the more aquatic ecology of *B.bombina* may be reflected in the number

of digenean metacercariae recovered, a total of 23 species as opposed to only 6 for *B.variegata*. Moreover, for all other types of helminth, it is the aquatic lowland species which harbours the greater diversity.

Few parasites recovered represent larval stages (i.e. cestode and nematode cysts) with both types at low prevalences and intensities of infection, thus the toads examined in this study may only rarely act as intermediate or paratenic hosts. This also suggests that they are not regular prey items. Where the toads represent the definitive host but the parasites were recovered at low prevalences and/or in small numbers (*D.subclavatus*, *O.filiformis*, *O.ranae*, *R.ranae*), no conclusions can be drawn concerning host genetic constitution or ecology. Those helminths transmitted by diet (*A.ranae*, *G.alobata*, *Haematoloechus sp.*, *H.androphora*, cestode cysts) may indicate a degree of niche separation between host genotypes, however on this basis, no statistically significant differences in distribution were found. The positively skewed distributions for *G.alobata*, *Haematoloechus sp.* and overdispersed distribution for *H.androphora* may be related to the chance encounter of infected prey rather than food preference. Water-borne infective stages (represented by *D.subclavatus* & *O.ranae*) may reflect ecological differences between hosts but again no statistically significant differences in distribution were found for *D.subclavatus*, and *O.ranae* was only recovered in small quantities. For both water and prey-borne stages transmission may be punctuated, depending on host behaviour and the biology of intermediate hosts. The resulting patterns of distribution may be complex, requiring detailed examination of all aspects of life-cycles and behaviour. Direct penetration of the

host, a method employed by the nematodes *C.ornata*, *O.filiformis* and *R.ranae*, may result in the possibility of almost continuous transmission. With such an infection mechanism it may be predicted that parasite distribution within the host population will approach a normal distribution, however, a number of other factors will influence the parasite population (host immunology, host behaviour etc: discussed in Chapter 9). It is the combination of these factors which are most probable explanation for the positively skewed distribution of *C.ornata* (Fig.10.3A) recorded in this study. Furthermore, with increasing host age (of which SVL and body weight were used as approximate indicators in this study), infection levels should increase. The positive correlation recorded for host body weight/SVL and *C.ornata* infection at site 5 requires further investigation with regards to possible differences between pure and hybrid genotypes. As noted in Chapters 4 & 8, skeletochronology could provide a more accurate method of ageing the host population, particularly since *B.bombina* may live for 20 years (Duellman & Trueb, 1986).

Grabda-Kazubska & Lewin (1989) recovered digenean metacercariae at lower levels than nematodes, acanthocephalans and adult digeneans. For *B.variegata*, the digenean *D.subclavatus*, nematode *O.bialata* and acanthocephalan *A.ranae* were found in ponds at lower elevations, which harboured a greater invertebrate fauna and more aquatic plants. Grabda-Kazubska & Lewin (1989) examined 20 hybrids, documenting a restricted fauna in comparison to the pure host specimens. It is possible that this finding may be explained by the fact that the hybrids were collected from a 'boggy meadow' which may not contain the correct intermediate

hosts. Barandun (1991) noted that during the breeding season, *B.variegata* males may remain in ponds from 1 week to 1 month. Therefore, mating success will also influence the degree of exposure to aquatic infective stages. In this study, sample size of female toads was restricted, which did not allow statistical comparison for sex related differences in behaviour and ecology. Although variation in infection levels could not be tested statistically, in all cases the males possessed a wider variety of helminths (Table 10.6).

As noted in the Introduction, Sage *et al.* (1986) and Moulia *et al.* (1991, 1993) have documented hybrid susceptibility to nematode and cestode infection in European mice. Coustau *et al.* (1991) and LeBrun *et al.* (1992) recorded a similar trend for a particular parental genotype (and their closely related hybrids) in mussel and fish hybrid zones respectively. In this study a high proportion of specimens possessed hybrid genotypes (36/110), however, they were distributed between 6 sites (only at site 5 did their numbers allow for statistical analysis). The helminths recovered in this study did not show any statistically significant differences in distribution between pure and hybrid hosts in sympatry. However, populations were found to differ significantly for *C.ornata* and *G.alobata* (individual Mann-Whitney tests). It was hoped to obtain further samples of pure host genotypes from outside the zone, but this was not possible. The present study has added a number of new parasite species records for *Bombina spp.* in Yugoslavia. The extensive literature pertaining to the parasites of European fire and yellow-bellied toads (see Table 10.1 & 2) also shows that all of the species recovered, with the sole exception of *G.alobata*, are broad spectrum, non-specific

generalists. Therefore, on the basis of the data presented here, there is no indication that there are changes in genetic predisposition to infection which are associated with the *B.bombina/B.variegata* hybrid zone.

10.6 References.

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Digenea (adults)	*B.b.*	Hyb.	*B.v.*
Brandesia turgida (Brandes, 1888) Stossich, 1899	-	-	+
Bundera luciopercae Muller, 1776	+	-	-
Cephalogonimus retusus (Dujardin, 1845) Odhner, 1910	-	-	+
Diplodiscus subclavatus Pallas, 1760	+	-	+
Doliosaccus rastellus (Olsson, 1876) Travassos, 1930	+	-	+
Gorgodera cygnoides (Zeder, 1800) Paul, 1934	+	-	+
Gorgodera varsoviensis Sinicyn, 1905	+	-	-
Gorgoderina alobata Lees & Mitchell, 1965	+	+	+
Gorgoderina vitelliloba (Olsson, 1876) Szmitzin, 1905	+	-	+
Haematoloechus abbreviatus Bychowsky, 1932	+	+	+
Haematoloechus asper Looss, 1899	+	-	+
Haematoloechus bombynae (Zeder, 1800) Yamaguti, 1971	+	-	+
Haematoloechus ellipticus Eckstein, 1922	+	-	-
Haematoloechus mazuromovici Boschkow, 1964	-	-	+
Haematoloechus schulezei Wundsch, 1911	-	-	+
Haematoloechus variegatus (Rudolphi, 1819) Looss, 1902	+	+	+
Haematoloechus v. abbreviatus Bychowsky, 1932	+	+	+
Haematoloechus vojtkovae Prokopic & Krivanec, 1974	+	-	-
Haematoloechus sp.	+	-	+
Halipegus kessleri Grebnitzki, 1872	+	-	-
Halipegus ovocaudatus (Vulpian, 1859) Looss, 1899	-	-	+
Halipegus sp.	+	-	-
Haplometra cylindracea (Zeder, 1800) Looss, 1899	+	-	+
Opisthoglyphe ranae (Frolich, 1791) Looss, 1899	+	-	+
Opisthoglyphe rastellus Olsson, 1876	+	-	-
Plagiorchis mentulatus (Rudolphi, 1819) Stossich, 1904	-	-	+
Pleurogenes claviger Rudolphi, 1819	+	-	+
Pleurogenoides medians (Olsson, 1876) Travassos, 1921	+	-	+
Pleurogenoides stromi Travassos, 1930	-	-	+
Prosotocus confusus (Looss, 1894) Looss, 1899	+	-	+
Skrjabinoeces ellipticus (Ekstein, 1922) Skrjabin & Antipin, 1962	+	-	-
Skrjabinoeces similus (Looss, 1899) Sudarikov, 1950	+	-	+

Table 10.1A The helminth fauna of *Bombina bombina, B.variegata* and their hybrids from collections made throughout their range: Digenea (adults).

Digenea (larvae)	*B.b.*	Hyb.	*B.v.*
Cathaemasia hians Rudolphi, 1809	+	-	-
Diplostomum excavata (Rudolphi, 1803) Hughes, 1929	+	-	-
Diplostomum spathaceum (Rudolphi, 1819) Braun, 1893	+	-	-
Diplostomum sp.	+	-	-
Distoma acervocalciferum Gastaldi, 1854	+	-	-
Echinostoma sp.	+	-	-
/Encyclometra colubrimurorum (Rudolphi, 1819) Dollfus, 1951	+	-	-
Euparyphium melis (Shrank, 1788) Dietz, 1909	+	-	-
Euryhelmis squamata (Rudolphi, 1819) Poche, 1925	-	-	+
Hypoderaeum conoideum (Bloch, 1782) Dietz, 1909	-	-	+
Isthmiophora melis Shrank, 1788	+	-	-
Leptophallus nigrovenosus (Bellingham, 1844) Luhe, 1909	+	-	+
Neodiplostomum spathoides Dubois, 1937	+	-	-
Opisthoglyphe ranae (Frolich, 1791) Looss, 1899	+	-	+
Paralepoderma cloacicola (Luhe, 1909) Dollfus, 1950	+	-	-
Psilochasmus sp.	+	-	-
Strigea falconis Szidat, 1928	+	-	-
Strigea sphaerula Rudolphi, 1803	+	-	-
Strigea strigis (Schrank, 1788) Abildgaard, 1970	+	-	-
Strigea sp.	+	-	-
Tetracotyle crystallina (Rudolphi, 1819) Linstow, 1877	+	-	-
Tetracotyle sp.	+	-	+
Tylodelphys excavata Rudolphi, 1803	+	-	-
Tylodelphys rhachidis Diesing, 1850	+	-	-
Trematode larvae	+	-	+

Table 10.1B The helminth fauna of *Bombina bombina*, *B.variegata* and their hybrids from collections made throughout their range: Digenea (larvae).

C.

Nematoda	*B.b.*	Hyb.	*B.v.*
Agamonema bombinatoris (Linst, 1892) Travassos, 1915	+	-	-
Aplectana acuminata (Schrank, 1788) Paul, 1934	+	-	-
Aplectana stromi Travassos, 1931	+	-	-
Cosmocerca commutata Diesling, 1851	+	-	+
Cosmocerca ornata Dujardin, 1845	+	+	+
Capillaria bombinatoris Linst, 1872	+	-	-
Heduris androphora Nitzsch, 1821	+	-	+
Neoraillietnema praeputiale Skrjabin, 1916	+	-	-
Oswaldocruzia bialata Molin, 1860	+	-	+
Oswaldocruzia filiformis Goeze, 1792	+	-	+
Oswaldocruzia goezei Skrjabin & Schultz, 1954	+	-	-
Oswaldocruzia subauricularis *	-	-	+
Oswaldocruzia ukrainae Iwanitzky, 1928	+	-	+
Oxysomatium brevcaudatum Zeder, 1800	+	-	-
Rhabdias bufonis Schrank, 1788	+	-	-

D.

	B.b.	Hyb.	*B.v.*
Monogenea			
Polystoma integerrimum (Frolich, 1791) Rudolphi, 1808	+	-	-
Acanthocephala			
Acanthocephalus falcatus Frolich, 1989	+	-	-
Acanthocephalus ranae Schrank, 1788	+	+	+
Centrorhynchus aluconis (Muller, 1780) Luhe, 1911	+	-	-
Cestoda			
Nematotaenia dispar Goeze, 1782	-	-	+
Tetrathyridium sp. Vojtkova, 1963	+	-	-

Table 10.1 The helminth fauna of *Bombina bombina*, *B.variegata* and their hybrids from collections made throughout their range: **C.** Nematoda, **D.** Monogenea, Acanthocephala & Cestoda. Complied from Govorka, (1988); Grabda-Kazubska & Lewin (1989); Matskasi *et al.*, (1990); Murai *et al.*, (1983, 1986); Prokopic & Krivanec (1974); Prudhoe & Bray (1982); Sey (1990); Sharpilo & Iskova (1989); Vojtek (1989); Vojtkova (1982) & Walton (1938). Designation of authorities based on source material. (Abbreviations: *B.b.* = *B.bombina*, *B.v.* = *B.variegata*, Hyb. = hybrid genotypes: * authority not quoted).

Trematoda.

Polystoma integerrimum (Frolich, 1791) Rudolphi, 1808

Cephalogonimus retusus (Dujardin, 1845) Odhner, 1910
Diplodiscus subclavatus Pallas, 1760
Dolichosaccus rastellus (Olsson, 1876) Travassos, 1930
Gorgodera cygnoides (Zeder, 1800) Paul, 1934
Gorgodera dollfusi Pigulevsky, 1945
Gorgodera sp.
Gorgoderina alobata Lees & Mitchell, 1966
Haematoloechus asper Looss, 1899
Haematoloechus variegatus (Rudolphi, 1819) Looss, 1902
Opisthoglyphe ranae (Frolich, 1791) Looss, 1899
Pleurogenes claviger Rudolphi, 1819
Pleurogenoides medians (Olsson, 1876) Travassos, 1921
Prosotocus fuelleborni Travassos, 1930

Trematode metacercariae.

Encyclometra sp.
Codonocephalus urnigerus Rudolphi, 1819
Neodiplostomum sp.

Cestoda.

Diphyllobothrium plerocercoids
Nematotaenia dispar Goeze, 1782

Acanthocephala.

Acanthocephalus ranae Schrank, 1788
Centrorhynchus aluconis (Muller, 1780) Luhe, 1911

Nematoda.

Aplectana acuminata (Shrank, 1788) Paul, 1934
Aplectana schneideri Travassos, 1931
Cosmocerca communata Diesling, 1851
Cosmocerca ornata Dujardin, 1845
Icosiella neglecta (Diesling, 1851) Seurat, 1917
Oswaldocruzia filiformis Goeze, 1792
Oswaldocruzia goezei Skrjabin & Schultz, 1954
Oxysomatidium brevicaudatum Zeder, 1800
Rhabdias bufonis Schrank, 1788

Table 10.2 The helminth fauna of amphibians from the former Yugoslavia.
Host species examined: *Bombina bombina* (n = 102); *B. variegata* (n = 200); *Bufo bufo* (n = 34); *B. viridis* (n = 855 ; *Hyla aborea* (n = 1); *Rana agilis* (n = 11); *R. esculenta* (n = 199); *R. ridibunda* (n = 849) and *R mp ria* (n = 231). Complied from records by Rozman (1976) and Vojtkova (1990).

	B.b.	Hyb.	*B.v.*
Diplodiscus subclavatus	+	+	+
Gorgoderina alobata	+	+	+
Haematoloechus sp.	+	+	+
Opisthoglyphe ranae	-	+	+
Acanthocephalus ranae	+	+	-
Larval cysts (Cestoda)	+	-	+
Cosmocerca ornata	+	+	+
Heduris androphora	+	+	+
Oswaldocruzia filiformis	-	+	+
Rhabdias bufonis	+	+	-
Encysted nematodes	-	+	+

Table 10.3 The parasite fauna of *Bombina bombina* (n = 29), *B.variegata* (n = 45) and their hybrids (n = 36) collected from 6 sites in the former Yugoslavia. (Abbreviations: *B.b.* = *B.bombina*; *B.v.* = *B.variegata*; Hyb. = hybrid genotypes; + = present; - = not recovered).

A.

Site	Host	n	*C.ornata* prev.	int.	*H.androphora* prev.	int.	*O.filiformis* prev.	int.	*R.bufonis* prev.	int.
1	Hyb.	9	55.5	2.8	22.2	4.0	11.1	10.0	-	-
2	*B.b.*	3	-	-	33.3	3.0	-	-	-	-
2	Hyb.	5	60.0	5.3	40.0	5.5	-	-	-	-
2	*B.v.*	44	86.4	3.2	-	-	4.5	6.5	-	-
3	*B.b.*	2	50.0	3.0	50.0	25.0	-	-	-	-
3	Hyb.	3	66.6	1.0	66.6	6.5	33.3	1.0	-	-
4	Hyb.	1	-	-	100.0	1.0	-	-	-	-
5	*B.b.*	24	33.3	2.5	33.3	2.5	-	-	4.2	1.0
5	Hyb.	15	46.7	5.4	46.7	5.4	6.7	1.0	6.7	1.0
6	*B.v.*	1	100.0	9.0	100.0	3.0	-	-	-	-
6	Hyb.	3	100.0	1.3	33.3	14.0	33.3	1.0	-	-

B.

Site	Host	n	*D.subclav.* prev.	int.	*G.alobata* prev.	int.	*Haemato.sp.* prev.	int.	*O.ranae* prev.	int.
1	Hyb.	9	-	-	3.0	2.0	-	-	22.2	1.0
2	*B.b.*	3	66.6	4.5	-	-	-	-	-	-
2	Hyb.	5	-	-	80.0	2.3	-	-	20.0	9.0
2	*B.v.*	44	4.5	81.5	52.3	2.4	20.5	2.8	6.8	1.3
3	*B.b.*	2	-	-	-	-	50.0	11.0	-	-
3	Hyb.	3	-	-	-	-	-	-	-	-
4	Hyb.	1	-	-	-	-	-	-	-	-
5	*B.b.*	24	12.5	3.3	12.5	1.7	16.7	3.0	-	-
5	Hyb.	15	26.7	32.5	13.3	1.5	26.7	1.3	-	-
6	*B.v.*	1	-	-	-	-	-	-	-	-
6	Hyb.	3	-	-	100.0	1.7	-	-	-	-

Table 10.4 Infection levels of A.) nematode and B.) digenean parasites of 110 specimens of *Bombina bombina*, *B.variegata* and their hybrids at 6 collection sites in the former Yugoslavia.

br n *B.b* = *B.bombina*; *B.v.* = *B.variegata*; Hyb. = hybrid genotypes; n = sample size; prev. = re alence; int. = mean intensity; collection sites and parasite species as described in the text, except f r *D.subclav.* = *D.subclavatus* & *Haemato.sp.* = *Haematoloechus sp.*).

A.

		B. variegata Males (n = 35)	Hybrids Males (n = 11)	*B. bombina* Males (n = 21)
SVL/mm	mean	43.7	41.3	40.1
	S.D.	3.4	2.3	4.1
	range	35.5 - 51.0	39.0 - 46.0	33.5 - 47.0
B wt/g	mean	8.6	7.0	6.7
	S.D.	1.9	0.9	1.8
	range	4.8 - 12.5	5.7 - 8.3	3.6 - 9.7

B.

		B. variegata Females (n = 9)	Hybrids Females (n = 4)	*B. bombina* Females (n = 3)
SVL/mm	mean	44.1	42.5	39.3
	S.D.	3.9	2.7	3.9
	range	38.0 - 49.5	39.0 - 46.0	34.0 - 43.0
B wt/g	mean	8.7	7.2	6.6
	S.D.	2.1	1.0	1.6
	range	5.9 - 12.2	5.8 - 8.5	4.5 - 8.3

Table 10.5 Snout-vent length (SVL) and total body weight (B wt) of **A.** male and **B.** female *B. variegata* (from site 2), *B. bombina* and hybrid genotypes (from site 5).

A.

	B.b. Male (n = 21)		*B.b.* Female (n = 3)		Hyb. Male (n = 11)		Hyb. Female (n = 4)	
	prev.	int.	prev.	int.	prev.	int.	prev.	int.
D.subclavatus	14.3	3.3	-	-	18.0	63.0	50.0	2.0
G.alobata	4.8	1.0	33.3	3.0	9.0	2.0	25.0	1.0
Haematoloechus sp.	9.5	1.0	-	-	27.3	1.3	25.0	1.0
O.ranae	-	-	-	-	-	-	-	-
A.ranae	23.8	1.6	33.3	5.0	9.0	6.0	25.0	1.0
C.ornata	76.2	3.3	100.0	3.3	63.6	2.3	75.0	3.3
H.androphora	33.3	3.0	66.7	6.5	45.5	4.2	50.0	8.5
O.filiformis	-	-	-	-	9.0	1.0	-	-

B.

	B.v. Male (n = 35)		*B.v.* Female (n = 9)	
	prev.	int.	prev.	int.
D.subclavatus	5.7	81.5	-	-
G.alobata	45.7	2.5	77.8	2.1
Haematoloechus sp.	22.9	3.0	11.1	1.0
O.ranae	8.6	1.3	11.1	1.0
A.ranae	-	-	-	-
C.ornata	91.4	3.1	66.7	4.0
H.androphora	-	-	-	-
O.filiformis	5.7	6.5	-	-

Table 10.6 Infection levels of the 8 most common parasites species by host gender and genotype at **A.**) site 5 and **B.**) site 2. (Abbreviations as described for Table 9.4).

A). *C.ornata*

	B.v.	Hybrids
B.b.	0.2810	0.2855
Hybrids	0.0352*	-

B). *D.subclavatus*

	B.v.	Hybrids
B.b.	0.6397	0.6249
Hybrids	0.3889	-

C). *G.alobata*

	B.v.	Hybrids
B.b.	0.0055*	0.9770
Hybrids	0.0216*	-

D). *Haematoloechus sp.*

	B.v.	Hybrids
B.b.	0.8074	0.6755
Hybrids	0.8210	-

E). *H.androphora*

	Hybrids
B.b.	0.5254

F). *A.ranae*

	Hybrids
B.b.	0.3408

Table 10.7 Individual Mann-Whitney tests of the distribution of the 6 most common parasites recovered from *B.variegata* (n = 44) from site 2, *B.bombina* (n =24) and ybrid genotypes (n = 15) from site 5. (Abbreviations: * = significant at or above the 95% confidence interval).

10.7 Legends.

Fig.10.1 The geographical distribution of *B.bombina* ▯ and *B.variegata* ▯ in Europe (adapted from Syzmura & Barton, 1991).

Fig.10.2 Frequency distribution of **A)** *G.alobata* and **B)** *Haematoloechus sp.* from *B.variegata* at site 2 (n = 44) and *B.bombina* (n = 24)/hybrid genotypes (n = 15) at site 5.

Fig.10.3 Frequency distribution of **A)** *C.ornata* and **B)** *H.androphora* from *B.variegata* at site 2 (n = 44) and *B.bombina* (n = 24) / hybrid genotypes (n = 15) at site 5.

Fig.10.4 Relationship between infection by *C.ornata* and host body weight in **A)** *B.bombina* (R^2 = 21.9%, F = 6.18, p = 0.021) and **B)** hybrid genotypes (R^2 = 38.2%, F = 8.03, p = 0.014).

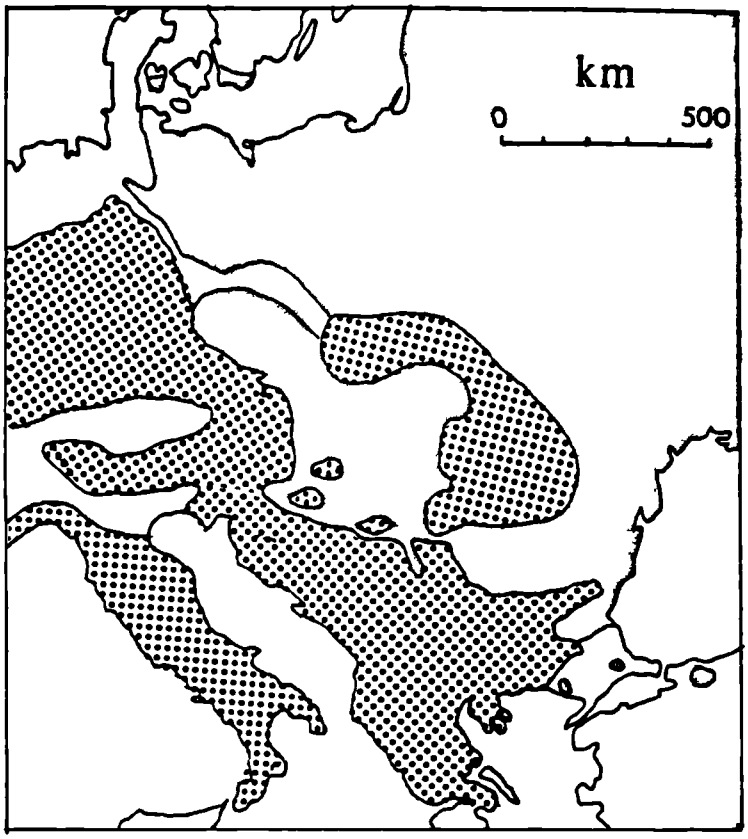
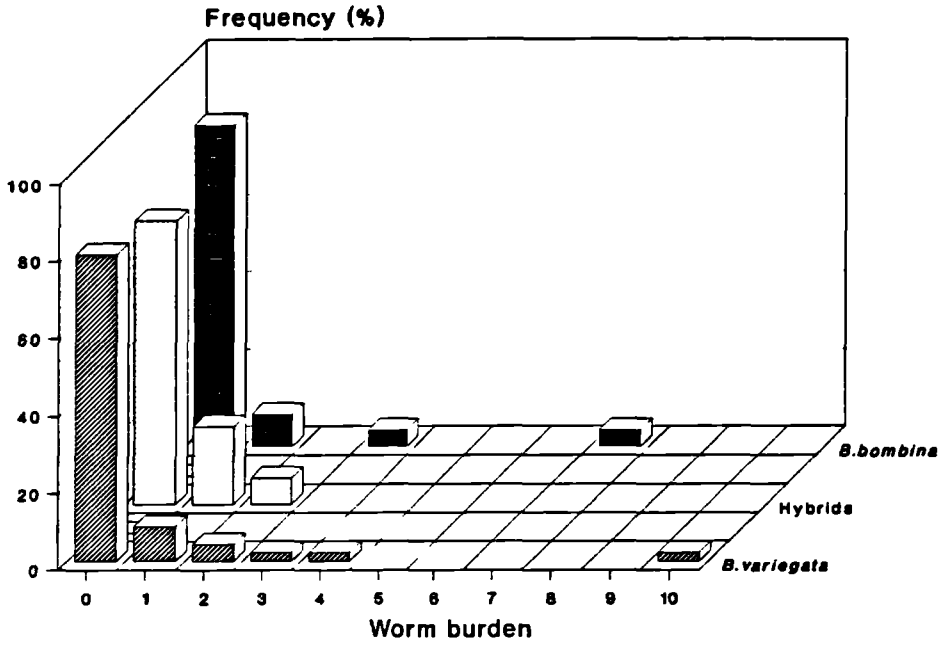


Fig.10.1

A


Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10
Worm burden
B.bombina
Hybrids
B.variegata

B

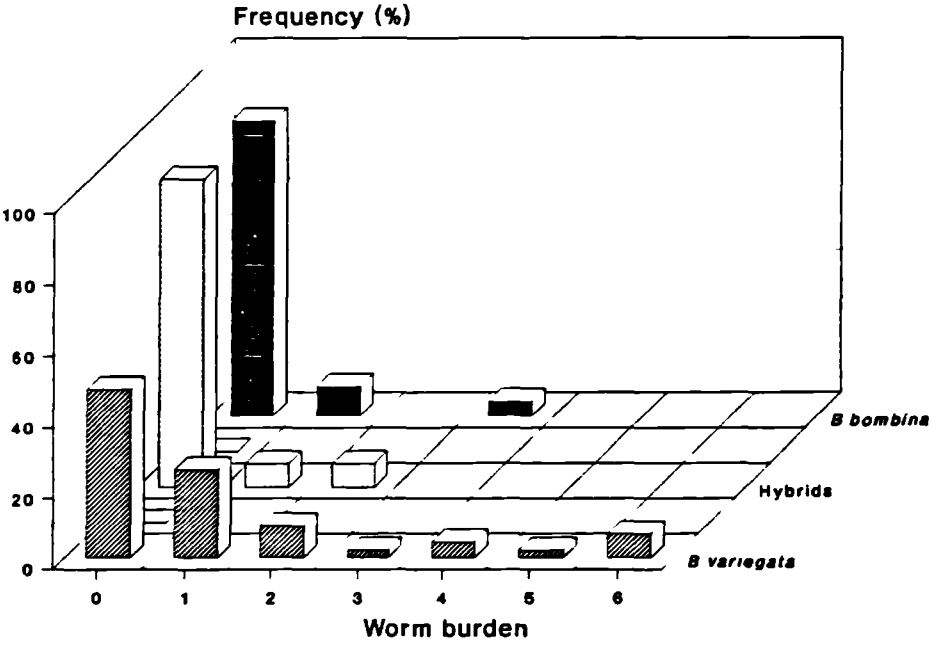
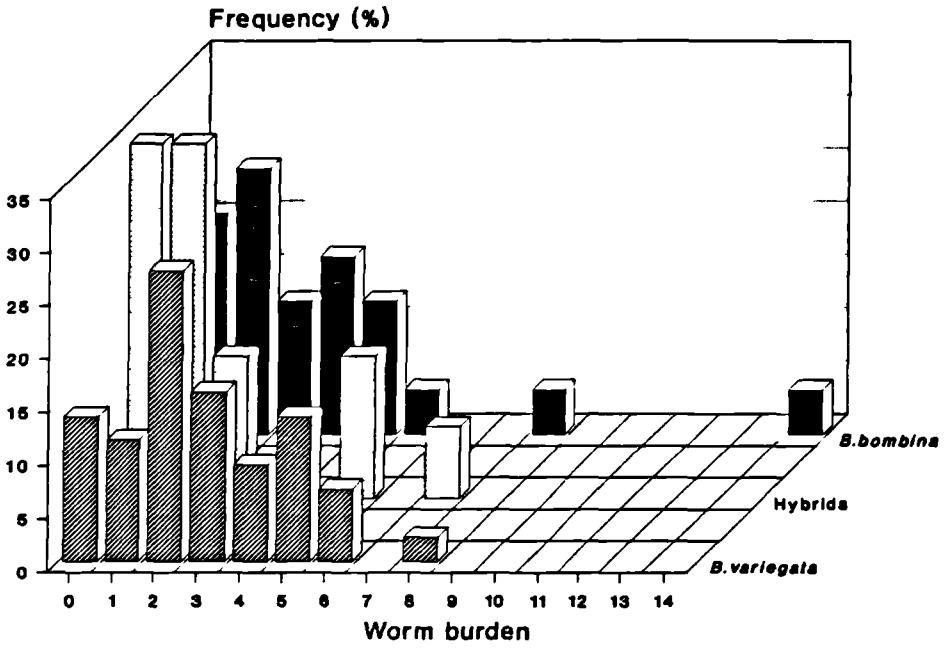

Frequency (%)
100
80
60
40
20
0
0 1 2 3 4 5 6
Worm burden
B bombina
Hybrids
B variegata

Fig.10.2

A


Frequency (%)
35
30
25
20
15
10
5
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14
Worm burden
B.bombina
Hybrida
B.variegata

B

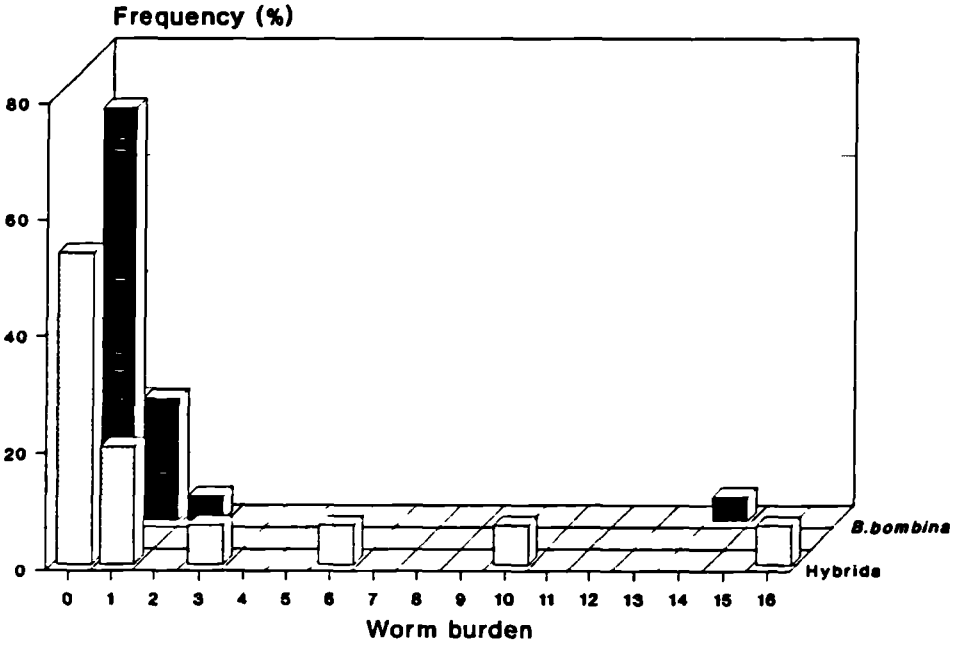
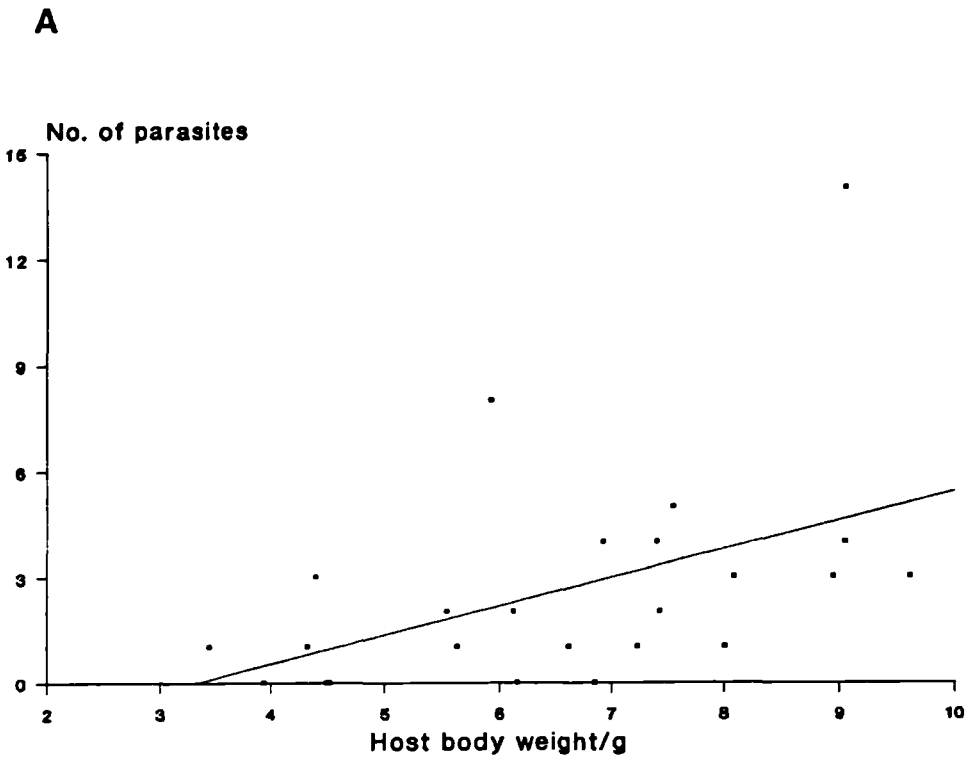

Frequency (%)
80
60
40
20
0
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
Worm burden
B.bombina
Hybrida

Fig.10.3


A
No. of parasites
15
12
9
6
3
0
2
3
4
5
6
7
8
9
10
Host body weight/g

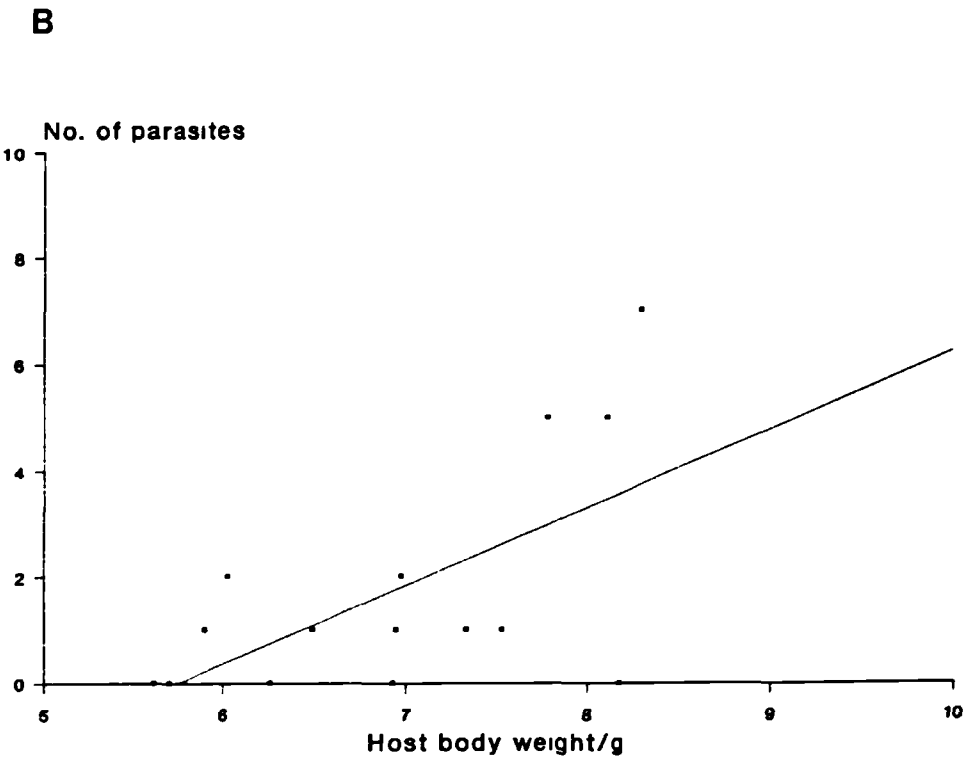

B
No. of parasites
10
8
6
4
2
0
5
6
7
8
9
10
Host body weight/g

Fig.10.4

Chapter 11.

11.1 Summary.

A detailed investigation of the parasitology of two North American anuran systems was undertaken in the laboratory and the field. The spadefoot toads, *Scaphiopus bombifrons* and *S.multiplicatus*, are closely related and may form hybrid zones in regions of sympatry. The polyploid complex of Gray treefrogs comprises a cryptic species pair; *Hyla chrysoscelis* being the diploid progenitor of the tetraploid *H.versicolor*. In this system, strong reproductive isolation maintains the integrity of each species. A parasitological survey was also undertaken on a third host system, the European fire and yellow-bellied toads, *Bombina bombina* and *B.variegata*. These closely-related toads form a narrow hybrid zone from Poland to the Black Sea, where elevations lie between the preferred habitats of pure populations. Specimens collected in the former Yugoslavia were studied.

By sampling *Scaphiopus* and *Bombina* in areas of sympatry, the distribution of helminths within parental genomes and an array of recombinant genotypes was examined. This provided an opportunity to examine whether there was any change in host susceptibility to parasitic infection. In the *Hyla* system, speciation is considered to have occurred via autopolyploidy, therefore species-specific parasites will have been confronted with the instantaneous doubling of the host's genome.

A second remit of this study concerned the polystomatid mongeneans, *Neodiplorchis scaphiopodis* (Rodgers, 1941) Yamaguti, 1963 infecting *S.bombifrons* and *S.multiplicatus*, and *Polystoma nearcticum* (Paul, 1935) Price, 1939 infecting members of the *H.chrysoscelis-H.versicolor* complex. *N.scaphiopodis* has been recorded from two host species and *P.nearcticum* has been reported to infect three host species. These records are of great interest as strict host specificity within the anuran Polystomatidae has been proposed by a number of authors. Therefore, the host systems not only allowed the testing of host susceptibility to parasitic infection but also monogenean specificity. In both the *Scaphiopus* and *Hyla* systems, it was conceivable that rather than a single species (*N.scaphiopodis* and *P.nearcticum* respectively) infecting both hosts, there could be separate cryptic, species-specific mongeneans.

In addition, there is little information in the literature pertaining to the post-oncomiracidial development of anuran polystomatids. Therefore, in addition to detailed morphological analysis of adult worms, the pre-migratory development of *N.scaphiopodis* and the neotenic development of *P.nearcticum* was investigated. Furthermore, parasites are typically overdispersed, generally fitting a negative binomial distribution. The causal factors in producing aggregated distributions may be mediated by both parasite and host, therefore, a further objective of this study aimed to assess the principal factors influencing the transmission dynamics of the *P.nearcticum-H.versicolor* system within controlled experimental infections.

The principal findings of this study are summarised below:

a. From the extensive surveys of all three host systems, parasite frequency distributions were typically aggregated for both pure-bred and hybrid hosts and there was no evidence of any change in genetic predisposition to infection. Apart from *N.scaphiopodis*, *P.nearcticum* and *G.alobata* (from *Bombina spp.*), the majority of species recovered were either broad spectrum generalists or utilised the anurans as intermediate/paratenic hosts. However, the surveys have documented a number of new host and/or parasite locality records for each host system.

b. The specificity of the monogeneans, *P.nearcticum* and *N.scaphiopodis*, was examined by extensive morphological analyses of the largest samples of adult worms yet described and by reciprocal cross-infection experiments. For *Polystoma*, morphological analyses did not separate worms from *H.chrysoscelis* and *H.versicolor*, suggesting that a single taxon, *P.nearcticum*, infects both members of this complex. This broader specificity was supported by preliminary cross-infections which indicated that branchial worms may develop on heterospecific tadpoles with an equivalent growth rate to conspecific infections. For *Neodiplorchis*, morphological analyses revealed no specific differences between worms from either *S.bombifrons* or *S.multiplicatus*. Moreover, cross-infection experiments have confirmed that *N.scaphiopodis* can reach sexual maturity and produce fully developed larvae in heterospecific hosts.

c. The neotenic development of *P.nearcticum* and the initial post-oncomiracidial stages of *N.scaphiopodis* were documented. The most significant finding from these investigations was the density-dependent egg production by neotenic *P.nearcticum*, which will have a strong influence on their contribution to transmission.

d. By controlling and/or moderating a number of factors in the experimental infection of *H.versicolor* tadpoles with *P.nearcticum*, a significant correlation was found between the mean worm burden/host and the initial oncomiracidial density. With a choice of hosts, the parasites tended to be distributed in a random to underdispersed manner rather than the aggregated distributions typical of natural parasite populations.

e. An unexpected finding of this study was the discovery of a digenean in a desert environment. It would appear that the manipulation of the environment by human activity has allowed the establishment of parasite species uncommon to that particular ecosystem. The introduction of molluscs and *Rana catesbeiana* into a man-made permanent pond in the Arizona desert has resulted in the infection of members of the autochthonous amphibian fauna (*Scaphiopus bombifrons* and *S.multiplicatus*) by the digenean *Clinostomum complanatum*.

f. In order to assess the genetic constitution of the spadefoot toads within the hybrid zone, it was necessary to electrophoretically-type each toad. This has provided data for the re-assessment of the hybrid-zone between *S.bombifrons* and

S.multiplicatus. This study recorded genotypic frequencies of adults comparable to those previously documented, which indicates that a region of 'balanced introgression' may exist. It would appear that introgression is primarily controlled by the prevailing weather, ecology and sexual behaviour of the host.